

Studies of opioid receptors and voltage-gated sodium channels on nociceptors in chronic inflammatory joint pain

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Declaration

I declare that the work in this thesis was carried out in accordance with the regulations stipulated by the University of Edinburgh. This thesis represents my own original work, with exception to the cases acknowledged below or by special reference in the text.

- The drug study performed in rats as part of the validation of the Pressure Application Measurement device was conducted by Dr. N.J. Barton and Mrs. S.M. Bond (The University of Edinburgh, UK).
- The effect of drugs on the heart rate and blood pressure measured during the *in vivo* electrophysiological experiments was analysed by Mrs. S.M. Bond (The University of Edinburgh, UK).

Signed .

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Associated abstracts and publications

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List of commonly used Abbreviations

°C	degrees Celsius	PBS	phosphate buffered saline
5-HT	5-hydroxytryptamine	PEG	polyethylene glycol
ANOVA	analysis of variance	PFA	paraformaldehyde
ASIC	acid sensing ion channel	PG	prostaglandin
ATP	adenosine triphosphate	PK	protein kinase
AUC	area under the curve	RA	rheumatoid arthritis
BK	bradykinin	s.c.	sub cutaneous
BP	blood pressure	SEM	standard error of the mean
cAMP	cyclic adenosine monophosphate	SP	substance P
CED	Cambridge Electronic Design	TNF α	tumor necrosis factor alpha
CGRP	calcitonin gene related peptide	TRP	transient receptor potential
COX	cyclo-oxygenase	TRPA	transient receptor potential ankyrin-type channel
dH ₂ O	distilled water	TRPM	transient receptor potential melastatin-type channel
DRG	dorsal root ganglion	TRPV	transient receptor potential vanilloid-type channel
EM1	endomorphin-1	TTX-r	tetrodotoxin resistant
EM2	endomorphin-2	TTX-s	tetrodotoxin sensitive
FB	Fast Blue	VGSC	voltage gated sodium channel
FCA	Freund's complete adjuvant	WHO	world health organisation
gf	gram force		
HLP	heavy liquid paraffin		
HPA	hypothalamic-pituitary axis		
i.a.	intra-arterial		
i.art.	intra-articular		
i.p.	intra-peritoneal		
i.v.	intra-venous		
IASP	International association for the study of pain		
IB4	isolectin B ₄		
IHC	immunohistochemistry		
IL	interleukin		
KD	knee diameter		
LWT	limb withdrawal threshold		
MAN	medial articular nerve		
mRNA	messenger RNA		
NGF	nerve growth factor		
NSAIDs	non-steroidal anti- inflammatory drugs		
OA	osteoarthritis		
OCT	optimum cutting temperature		
<i>P</i>	statistical probability		
PAM	pressure application measurement		
PAN	posterior articular nerve		

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Abstract

Joint inflammation during arthritis occurs initially as a result of damaged cells activating or releasing pro-inflammatory mediators. The subsequent infiltration of numerous inflammatory cells into the area of tissue damage further exacerbates the condition. The underlying pathophysiology of chronic inflammatory joint disease results in peripheral sensitisation of nerves at the site of the damaged tissue, together with central sensitisation of the spinal processes connected to that tissue, which leads to the pain that is a common distressing symptom of arthritis.

Most arthritis is chronic by the time a patient presents in the clinic, so a rodent model of chronic inflammatory joint pain was used to investigate the mechanisms which contribute to pain in human joints. Freund's complete adjuvant (FCA)-induced unilateral arthritis of the rat knee joint is a well established model of arthritis, mimicking the histopathology, hyperalgesia and swelling of the joint as seen in the clinic. *In vivo* electrophysiology, behaviour and immunohistochemistry techniques were used to elucidate the role played by opioid receptors and their endogenous ligands, and also the voltage-gated sodium channels (VGSCs), in chronic inflammatory joint hypersensitivity.

Exogenous opioids such as morphine have long been used in the management of pain as they exhibit powerful analgesic properties, which are generally attributed to centrally mediated actions. The peripheral effect of the recently discovered endogenous opioid peptide endomorphin-1 (EM1) and morphine was assessed using behavioural and electrophysiology techniques during the "chronic" inflammatory phase (14-28 days post-FCA) of the rat model of unilateral arthritis.

The effect of administering these opioid receptor agonists directly into the knee joint on behavioural measures of hyperalgesia was investigated to determine whether they provide analgesia when restricted to the periphery. Both EM1 and morphine significantly reduced joint hypersensitivity in a dose-dependent manner, as measured using an established readout of "pain", based on how the conscious animals distribute their weight between the inflamed and non-inflamed hind limbs.

The antinociceptive properties of EM1 and morphine were studied in rats anaesthetized with pentobarbital by recording the neuronal discharge patterns of primary afferent nerves innervating the rat knee joint evoked by mechanical and chemical stimulation. In arthritic animals both morphine and EM1 decreased neuronal firing evoked by mechanical (von Frey filament) stimulation of the inflamed knee, an effect that was abolished by pre-treatment with the opioid antagonist naloxone. In contrast, only morphine decreased the neuronal discharge evoked by capsaicin in both naïve and arthritic animals.

The VGSCs are crucial for transmitting information concerning environmental stimuli from the periphery to the central nervous system, and hence play an integral role in regulating the excitability of nociceptive primary afferent neurones. The expression levels of the VGSC's $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$, predominantly found in nociceptive primary afferents, were investigated in the cell bodies of the knee joint afferent population using immunohistochemical techniques. Expression was measured in naïve animals and also on days 7, 14, 21 and 28 days during the progression of the adjuvant-induced arthritis. $Na_v1.9$ expression was upregulated at days 14, 21 and 28 post-FCA, with $Na_v1.7$ and $Na_v1.8$ showing increased channel expression at days 14 and 28 post-FCA in comparison with time matched control animals.

Throughout the studies the effect of FCA and test compounds on the behavioural hypersensitivity and swelling of the knee joints was measured. A new device, designed to assess knee joint hypersensitivity directly across the knee joint of rodents, was developed and validated to help align pre-clinical measures with those used in the clinic with the view to help successfully correlate animal studies to human. The pressure application measurement (PAM) device consists of a recording base unit with digital display that is connected to a force transducer embedded in a moulded plastic thumb unit. A gradually increasing squeeze is applied across the knee joint between the thumb unit and forefinger and at the point the force applied across the knee became noxious, the animal withdraws its leg indicating the test endpoint. PAM recorded a joint hypersensitivity as shown by a

decrease in limb withdrawal threshold in FCA-injected animals. Equally PAM measurements showed the expected analgesic effect of prednisolone in both species, and also the attenuation of FCA-induced hypersensitivity in rats dosed with morphine or celecoxib. PAM is therefore a novel behavioural tool for detecting localised primary mechanical hypersensitivity in a model of chronic inflammatory joint pain in rodents.

In summary, results from the *in vivo* electrophysiology studies show that during the chronic phase of FCA-induced joint hypersensitivity there are functional opioid receptors present in the periphery and agonists at these receptors are able to attenuate nociceptive firing evoked by noxious mechanical von Frey filaments. Furthermore EM1 and morphine administered directly into the knee joint attenuate the FCA-induced joint hypersensitivity by activating opioid receptors located in the periphery. Immunohistochemical results showed that the number of primary afferent neurons expressing the VGSCs was upregulated during FCA-induced inflammation, which could account in some part for the increased neuronal hypersensitivity observed in chronic inflammatory joint pain. Future novel analgesics targeting opioid receptors and VGSCs in the periphery may facilitate the breakthrough of a novel potent analgesic drug without undesirable centrally mediated side-effects.

Chapter 1
General Introduction

The work presented in this thesis concerns the role that the opioid receptors and the VGSCs play in generating the chronic pain associated with inflammatory arthritis in the knee joint. By using behavioural; *in vivo* electrophysiological and immunohistochemical staining techniques, in a rat model of inflammatory knee joint monoarthritis, the mechanisms involved in the sensitisation of peripheral nociceptors were investigated.

This chapter provides an overview of the historical background and more recent understanding of the subject matter investigated throughout the thesis. The peripheral and central mechanisms involved in joint inflammation and nociception and how these processes commonly present themselves in the clinic in arthritic joint disease, along with commonly used experimental animal models of joint pain will be discussed. In addition the role of various inflammatory mediators, receptors and channels involved in these processes, along with potential pharmacological therapeutic agents will be considered, with particular emphasis on the opioid receptors and their endogenous ligands, and the VGSCs.

1.1 Clinical presentation of chronic pain

1.1.1 Introduction

Chronic pain is a major health problem that affects 19% of European adults in a moderate or severe form (Breivik et al. 2006), and is often presented in association with diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA). Chronic pain is ranked by the World Health Organisation (WHO) as one of the major reasons for health related work absence (Garcia and Altman 1997; Gureje et al. 1998). Pain is categorised as being chronic when symptoms are still present even after the resolution of the initial injury, or in degenerative diseases such as OA where the condition will never heal. It is not the duration that makes a pain chronic, but the inability of the body to return its physiological functions to normal. Chronic pain can lead to a severe reduction in the sufferers' quality of life caused by

psychological and social problems as a result of sleep disturbance, anxiety, depression and decreased functional capacity (Ashburn and Staats 1999).

There is great demand for the improved management of pain, but despite our increased understanding of the mechanisms involved in pain processing and the pathophysiology of diseases such as OA and RA, many patients still receive inadequate treatment. Pharmacological management has scarcely progressed in over a century, with unvarying reliance on cyclo-oxygenase (COX) inhibitors and the opioid analgesics (Rasor and Harris 2007). The recent withdrawal of Merck's COX-2 inhibitor, rofecoxib (Vioxx) due to several deaths from cardiovascular complications has eliminated one of the most effective and popular treatments available to patients (Fitzgerald 2002; Dieppe et al. 2004; Fitzgerald 2004). This has left a huge gap in the market and the race is on to find a novel analgesic without detrimental side effects (Mount and Featherstone 2005).

1.1.2 Arthritis

Arthritis is a term used for over 100 different conditions, with the most commonly occurring being OA and RA, each of which is considered below. The Centres for Disease Control report that in the US alone there are currently 46 million adults diagnosed with arthritis, with the prediction that this value will increase to 67 million by 2030. Arthritis is one of the most common conditions that gives rise to chronic pain (Fitzcharles et al. 2004). The tissue damage and inflammatory processes commonly involved in these two diseases result in the associated pain being described as both chronic and inflammatory.

1.1.2.1 Rheumatoid arthritis

RA is a prevalent, hostile autoimmune disease with unknown aetiology characterised by persistent synovitis and progressive deterioration of the articular joints. The incidence

rates in the UK are 1.16% in women and 0.44% in men, with women three times more susceptible to the condition than men (Symmons et al. 2002). It is thought that a combination of genetic factors, infectious agents and sex hormones trigger the autoimmune pathogenic pathway leading to articular cartilage loss and erosion of articular bone (Ollier et al. 2001). Increased understanding of the immune system means that the pathogenesis of RA has recently become clearer. A large amount of published evidence suggests that T lymphocytes, cytokines and macrophages play a critical role in the initiation and perpetuation of synovial inflammation (see Firestein 2003, for review), with these processes leading to joint pain, a loss in the range of movement and deformity in the limbs. RA a systemic disease often affecting tissues other than the joints, commonly including; the skin, lungs and muscles (Scott 2006). At the time of first diagnosis it is usual for just one joint to be affected with the disease commonly spreading to the corresponding contralateral joint as the disease progresses. Patients suffering from RA rate reducing the joint pain associated with their condition as being the most desirable outcome of their treatment (Heiberg and Kvien 2002; Heiberg et al. 2005).

1.1.2.2 Osteoarthritis

OA is the most common form of arthritis and results from the wear and tear of the joint rather than the autoimmune response attributed to RA (Felson and Zhang 1998). It is diagnosed by swollen stiff joints and a reduction in functionality. Commonly found in load bearing joints the condition involves cartilage loss, increased bone density in the subchondral region, cysts and formation of new bone and cartilage at the joint margin (Swagerty and Hellinger 2001). In well advanced cases of OA the cartilage can be completely missing causing the two ends of the bone to make contact and rub against each other. A single joint can be involved, but more commonly several joints including the knees, hips, hands and

spine are involved (Dieppe and Lohmander 2005). As with RA, pain is the dominant symptom for most individuals suffering from OA (Gooberman-Hill et al. 2007).

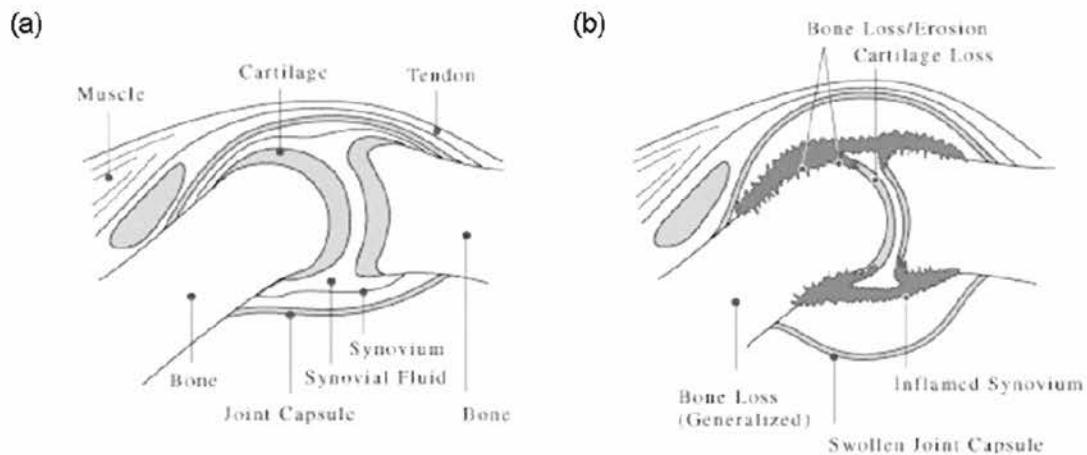


Figure 1.1 Diagram comparing the tissues in a healthy (a) and a rheumatoid arthritic (b) knee joint. The healthy joint has a thin smooth synovial lining whereas the synovium in the arthritic joint is inflamed and thickened due to the infiltration of inflammatory cells. Bone and cartilage loss are also evident in the arthritic joint. (Adapted from the National Institutes of Health, 2005).

1.1.3 Treatments currently available for chronic joint pain

Mild to moderate pain associated with RA and OA is usually treated with non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, diclofenac and ibuprofen which reduce pain and inflammation (Morgan et al. 1993). These drugs work by inhibiting the action of COX, thereby preventing the metabolism of arachadonic acid and consequently the production of the eicosanoids (Vane 1971). The eicosanoids play an integral role in the bodies inflammatory response, with prostaglandin E_2 (PGE_2) in particular being an important sensitising agent and mediator of pain (Katori 1989). The prolonged use of these drugs has been shown to have undesirable side effects such as reducing renal function (Lifschitz 1983) and causing gastrointestinal ulceration and bleeding (Goodwin 1987). The development of selective COX-2 inhibitors such as rofecoxib, celecoxib and etoricoxib potentially removed these side-effects which are mediated by inhibition of the COX-1 enzyme (Lipsky and Isakson 1997; Hawkey 1999; Riendeau et al. 2001). As mentioned above the COX-2

inhibitor rofecoxib has been cited in causing an increased risk of heart attack and stroke, which lead to the withdrawal of the drug in 2004. Rofecoxib was one of the most widely used drugs ever to be withdrawn from the international market and in 2003, before its withdrawal, Merck reported that sales of the drug amounted to 2.5 billion US dollars (New York Times, 07/12/2004).

Opioids such as morphine and oxycodone are potent analgesics that provide excellent relief in cases of severe acute and chronic pain (Dellemijn 2001; Przewlocki and Przewlocka 2001). Their long-term use is compromised by various unwanted side effects such as tolerance, addiction, constipation, nausea, respiratory depression and euphoria meaning that prescription of these drugs is often restricted (Nicholson 2003). These side effects appear to be mediated through centrally located opioid receptors and consequently there is great interest in opioids that selectively target receptors expressed in the periphery (Stein et al. 1989).

Recent clinical advances in the pharmacological treatment of pain have included the use of the anticonvulsant drug gabapentin. Gabapentin was originally introduced for the treatment of epilepsy (Crawford et al. 1987), but is now widely used in the treatment of post-operative and chronic pain (Guindon et al. 2007). Several hypotheses have been put forward suggesting its mechanism of action, but it is predominantly thought that its binding to a subunit of the voltage gated N-type calcium channel plays a key role in its analgesic properties (Cheng and Chiou 2006).

1.2 Pain and Nociception

1.2.1 Overview of pain

Pain is defined by the International Association for the Study of Pain (IASP) as; an unpleasant sensory and emotional experience associated with actual or potential tissue

damage, or described in terms of such damage. The sensation of pain is the body's first line of defence against any potentially threatening or damaging environmental stimuli. The pain sensation is essential for maintaining the health of the human body, with its two main functions being to recognise harmful stimuli and generate the reflex required to avoid tissue damage and to provide a means of sensing when damage has occurred enabling protection and repair of that damage.

1.2.2 The discovery of nociceptors

René Descartes is regarded as the first philosopher to hypothesize correctly that pain results from peripheral nerves transmitting information to the higher centres in the brain (see Figure 1.2). Early scientists accepted that pain was a sensation in its own right, but did not appreciate that it was detected by its own special sense organs (Perl 1996). This was first described by the Austrian physiologist Max von Frey who revealed that the skin was comprised of an intricate mosaic of minute sensory areas relating to touch, coldness, warmth and pain, all mediated by specific sensory organs (von Frey 1894; 1922). From this he deduced the morphological type of specialized afferent nerve terminals related to each type of sensation, and he attributed pain to fine unspecialised free nerve endings.

In 1903 Charles Sherrington further described how different kinds of nerve endings exist in the skin of dogs, with one of his perceptive remarks being "There is considerable evidence that the skin is provided with a set of nerve-endings whose specific office it is to be amenable to stimuli that do the skin injury, stimuli that in continuing to act would injure it still further.", he further went on to infer that "the species of nerve-ending excited in these cases may preferably be termed nocicipient" (Sherrington 1903). Sherrington's work on mammalian neuronal function, summarised in his book, *The Integrative action of the nervous system*, greatly developed the understanding of nervous function and pain sensation (Sherrington 1906). Sherrington argued that pain is a specific sensation with its own sensory

machinery which he named 'nociceptors'. First published over 100 years ago, this work provided a framework for subsequent research that led to our modern day understanding of the mechanisms involved in synaptic transmission and pain (Burke 2007; Levine 2007).



Figure 1.2 Descartes model of pain sensation showing the hypothesised link between the peripheral tissues and the brain in sensing painful stimuli such as noxious heat. This cartoon, adapted from the original published by Descartes in his 1606 work entitled *L'Homme*, is considered to be one of the earliest systematic representations of the organization whereby a noxious stimulus activates systems that travel through the nerves to the spinal cord and then to the brain.

The introduction of electrophysiology studies capable of recording the compound action potentials of electrical nerve signals (Adrian 1926a; b; Adrian and Zotterman 1926) showed that peripheral nerves were composed of groups of fibres conducting at different velocities (Erlanger and Gasser 1937). Groups of fibres were named in the order in which their signal appeared on the oscilloscope screen after a brief single electrical impulse given to the nerve trunk. Subsequent investigations were undertaken into how the different fibre types responded to various stimuli (Heinbecker et al. 1932; Zotterman 1933; Clarke et al. 1935). In mammals, A β -fibres are the fast conducting ($35\text{-}100\text{m.s}^{-1}$), large diameter ($>10\mu\text{m}$) myelinated fibres that respond to light touch and innocuous stimuli. A δ -fibres are slower conducting ($5\text{-}30\text{m.s}^{-1}$) medium diameter ($2\text{-}5\mu\text{m}$) myelinated fibres and C-fibres are the slow conducting ($0.05\text{-}2\text{m.s}^{-1}$), small diameter ($0.2\text{-}1.5\mu\text{m}$) unmyelinated fibres. A δ - and C-fibres both respond to noxious stimuli. The development of a micropipette recording

technique in 1965 allowed characterisation of 513 cutaneous afferents, recorded in the anaesthetised cat, to thermal and mechanical stimulation and showed a significant population of A δ -fibres that were activated exclusively by noxious mechanical stimuli (Burgess and Perl 1967). These afferents were named nociceptors, a term derived from Sherrington's earlier work. In experiments on the skin innervating the hind limb of a cat the unmyelinated C-fibres were later described as being either low-threshold afferents that were activated by innocuous mechanical stimuli, or high-threshold nociceptive afferents that were responsive to noxious mechanical stimuli, high temperatures and chemical stimulation (Bessou and Perl 1969). Of the high-threshold nociceptors approximately one third were responsive to intense mechanical stimulation only and two-thirds were responsive to all the noxious stimuli. The former group of nociceptors were named mechanonociceptors and the later were named polymodal nociceptors.

1.2.3 Current understanding of afferent nerve fibres

The nociceptive primary afferent neurone has four major components; the peripheral terminal, the axon, the cell body and the central terminal (Figure 1.3a). The normal resting membrane potential of primary afferents is typically -70mV. The peripheral terminal transduces the external stimuli into an action potential as a consequence of the terminal becoming depolarised as a result of various ion channels being activated, which further trigger the opening of voltage gated sodium channels (VGSCs Figure 1.3b). A wave of depolarisation occurs as a result of the increased permeability of the cell membrane to sodium and potassium ions (Hodgkin and Huxley 1952a; b; c). Action potentials are propagated along the axon to the central terminal in the dorsal horn of the spinal cord which forms the presynaptic component of the first synapse in the sensory pathway. Calcium ions enter the central terminal and cause the release of both inhibitory and excitatory neurotransmitters as summarised schematically in Figure 1.3c.

The classification of a primary afferent as being either an A β -, A δ - or C-fibre is still determined by conduction velocity and axonal diameter, with only the A δ - and C-fibre types considered to be involved in nociceptive signalling (Dubner 1978; Handwerker and Kobal 1993). Within these groups further subtypes exist, as determined by their sensory profile and the molecular markers which they express. Activation of A δ -fibres evokes sharp acute pain, a prickling sensation and aching pain. Two sub sets of A δ -fibres are apparent; type I and type II with type I being the most common. Type I fibres typically respond to heat, chemical and mechanical stimuli whereas type II are mechanically insensitive (Treede et al. 1995; Lawson 2002). As discussed above, C-fibres can be described as either polymodal or mechanonociceptors (Bessou and Perl 1969). Another population of C-fibres are the silent or mechanically insensitive afferents, first described in the knee joints these sensory afferents are present throughout the periphery (Schaible and Grubb 1993). Silent nociceptors are normally unresponsive to acute noxious stimuli, but following tissue damage and inflammation the inflammatory mediators lead to a decrease in the activation threshold of these nociceptors rendering them active to noxious stimuli (McMahon and Koltzenburg 1990; Cervero et al. 1994).

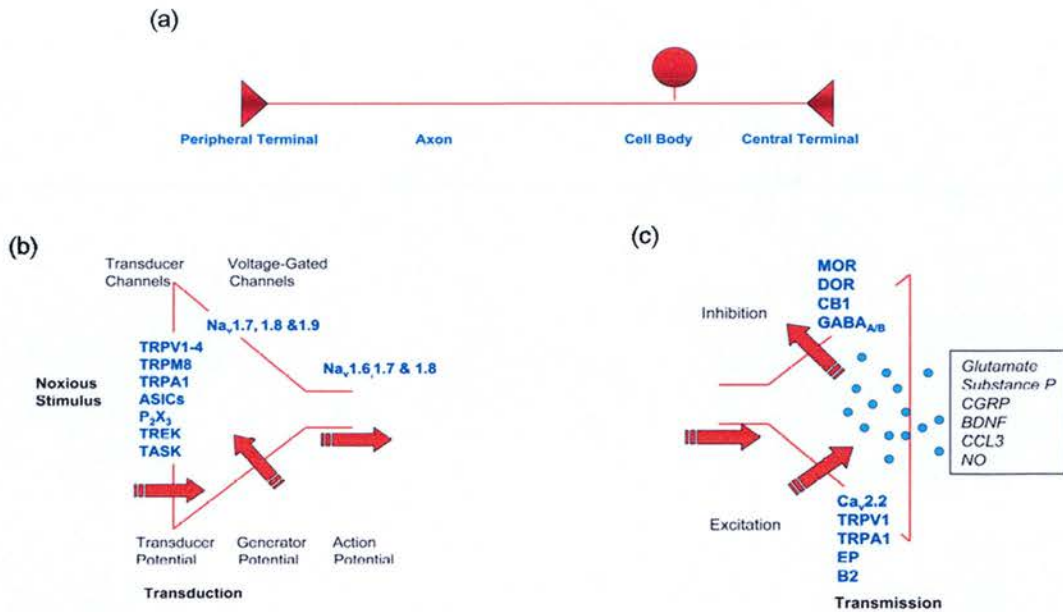


Figure 1.3 (a) Basic structure and components of a primary afferent neurone. The peripheral terminal innervates the target tissue, the axon conducts action potentials and the central terminal transfers the signal to central processes. The cell body controls the identity of the nerve and produces the proteins the receptors and channels that can be transported to the peripheral or central terminal. Schematics showing (b) a selection of ion channels involved in the depolarisation of the peripheral terminal and (c) neurotransmitters released from the central terminal. Diagram modified from (Woolf and Ma 2007).

Neurochemical and electrophysiological studies in both mice and rats have revealed that two distinct subsets of nociceptive C-fibres exist that are determined by the ion channels and receptors they express and their central projections in the superficial dorsal horn (Hunt and Rossi 1985; Stucky and Lewin 1999; Braz et al. 2005). The first subset express the NGF receptor TrkA and rely on nerve growth factor (NGF) for their survival; these nociceptors contain the peptides substance P (SP) and calcitonin gene related peptide (CGRP) and are therefore termed peptidergic (Molliver et al. 1995; Bennett et al. 1996). The peptidergic nociceptors project to lamina I and the outer layer of lamina II in the dorsal horn (Zylka et al. 2005). The second class of nociceptors are determined by the expression of isolectin B4 (IB4) and do not contain SP or CGRP and are therefore named non-peptidergic or IB4-positive (Molliver et al. 1997). The non-peptidergic nociceptors project preferentially to the inner layer of lamina II of the dorsal horn (Hunt and Rossi 1985). In rats peptidergic and

non-peptidergic neurones express different patterns of receptors and ion channels, for example the adenosine triphosphate (ATP) receptor P2X₃ is expressed primarily in non-peptidergic (Vulchanova et al. 1998) whereas the transient receptor potential vanilloid 1 (TRPV1) receptor is expressed equally in both types (Caterina et al. 1997). It is thought that peptidergic neurones are the main subset involved in generating neurogenic inflammation by releasing SP from their peripheral terminals in response to pro-inflammatory stimuli (De Felipe et al. 1998). This process is considered to initiate chronic pain following tissue damage (Woolf et al. 1998). Non-peptidergic neurones have been implicated in the generation of chronic pain following nerve damage (Malmberg et al. 1997).

1.2.4 Pain sensation (transduction)

Pressure, light touch, chemical and thermal (hot or cold) stimuli can be transduced by the peripheral terminal of afferent neurones into an electrical signal (action potential) which is transmitted to the central nervous system along the nerve axon, as described above. Stimulation of the skin by chemical irritants produces an acutely painful response, but this is not a model relevant to chronic inflammatory pain as studied in this thesis. However, chemical mediators such as SP and histamine do play an important role in neurogenic inflammation and peripheral sensitisation following tissue injury. The chemical mediators can be released from the afferents themselves, mast cells, surrounding tissue including the vasculature and inflammatory cells. These chemical mediators and their role in sensitising peripheral nerves will be discussed further in section 1.3.2.

1.2.4.1 Mechanical stimuli

Relatively little is known about the mechanisms involved in detecting noxious mechanical stimuli. Much of our understanding comes from studies into the bacterial

mechanosensitive ion channels which show both large and small conductance (see Corry and Martinac 2007 for review). Recent studies have highlighted the role of neurotrophin-3 (McIlwrath et al. 2007) and endothelin-1 (Namer et al. 2007) in regulating the mechanical sensitivity of primary afferents. Several ion channels have been implicated in the mechanosensitivity of mammalian afferents (see Sukharev and Corey 2004 for review). The acid sensing ion channel (ASIC), transient receptor potential family (TRPA1) and P2Y receptors have all been suggested to play an important role in sensing mechanical stimuli (Nakamura and Strittmatter 1996; Corey et al. 2004; Kwan et al. 2006; Lingueglia 2007).

1.2.4.2 Thermal stimuli

Detection of thermal sensation such as heat or cold allows an organism to regulate their internal environment. In the monkey, temperatures below 15°C and above 43°C illicit neural discharge and a subsequent thermal sensation (LaMotte and Campbell 1978; Tillman et al. 1995), and a specific heat gated ion channel was identified in a subset of primary afferent neurones (Cesare and McNaughton 1996; Reichling and Levine 1997). The cloning of the TRPV1 receptor in 1997 really progressed our understanding of the ion channels involved in painful heat sensation (Caterina et al. 1997). Early behavioural studies in rats were the first to suggest that the burning sensation caused by application of capsaicin to the skin was mediated through such a receptor (Szolcsanyi and Jancso-Gabor 1975; Szolcsanyi et al. 1975). Capsaicin, the component of chilli peppers from the genus *Capsicum* that makes the pepper 'hot', was identified as an exogenous ligand to these receptors, being able to evoke neuronal discharge in a population of sensory afferents and cause the release of the inflammatory mediators SP and CGRP (Szolcsanyi et al. 1988; Szallasi and Blumberg 1999). The TRPV1 receptor has a threshold of activation of approximately 43°C (Caterina and Julius 2001). The receptor is not only activated by heat and capsaicin, but also by protons present in greater concentrations than usual at a pH of <6. To date the cloning of the TRP

family of receptors has revealed 3 subtypes, the TRPV subtype made up of TRPV1-TRPV6, the TRPM family comprising TRPM1-8 and the TRPA family formed from the TRPA1 receptor only (Tominaga and Caterina 2004). Of these receptors, activation of TRPV1, TRPV2, TRPV3 and TRPV4 occurs as a result of warm to hot temperature conditions or stimuli (Jordt et al. 2003), with TRPM8 and TRPA1 being activated by cold stimuli or temperatures below 15°C (Campero et al. 1996).

TRPV Family	TRPM Family	
TRPV1	TRPM1	TRPM5
TRPV2	TRPM2	TRPM6
TRPV3	TRPM3	TRPM7
TRPV4	TRPM4a	TRPM8
TRPV5	TRPM4b	
TRPV6		

Table 1.1 Members of the TRPV and TRPM family. Red, orange, and blue text indicates the channels which are activated by high heat (>43°C), warm (>30°C), and cold (<15°C) stimuli respectively.

TRPV1 expression studies have shown the receptor to be most densely expressed in small diameter cell bodies which give rise to unmyelinated C-fibres, and centrally in lamina I and II of the dorsal horn where these unmyelinated C-fibres project. Deletion of the TRPV1 receptor in knockout mice caused significant deficits in thermal sensation at both peripheral and central aspects of the pain pathway but these mice were not completely insensitive to thermal nociception, suggesting that TRPV1 mediates some but not all thermal sensation (Caterina et al. 2000). Mediators such as ATP and bradykinin have been shown to lower the temperature threshold for activation of TRPV1 receptors to as low as 30°C through protein kinase mediated phosphorylation of the receptor suggesting that inflammatory pain may

occur as a result of modulation of this receptor (Reeh and Petho 2000; Numazaki et al. 2002).

The methods involved in the transduction of noxious cold sensation are less well studied than those involved in noxious heat. The cooling sensations of low temperatures and menthol were described in the early 1950's (Hensel and Zotterman 1951), with the sensations later being attributed to a non selective cation cold receptor called TRPM8 (McKemy et al. 2002; Peier et al. 2002). TRPM8 is normally activated by temperatures lower than 25°C but as described above with TRPV1, modulation of the channel can lead to sensitisation of the channel, with its activation being seen at temperatures up to 30°C (McKemy et al. 2002; Peier et al. 2002). Like TRPV1, TRPM8 is expressed in small-diameter C-fibres, yet the two receptors do not appear to ever be co-expressed on the same neurones (Nealen et al. 2003).

In addition to TRPM8, the TRPA1 receptor has been shown to be involved in cold sensation with a lower activation threshold than TRPM8 (Story et al. 2003). The receptor is activated at temperatures below 17°C and unlike TRPM8 menthol is not a ligand for this receptor. TRPA1 is expressed exclusively in peptidergic neurones and is often seen co-expressed with TRPV1. TRPA1 knockout mice appear to be deficient in detecting noxious cold pain sensations at temperatures less than 0°C, but exhibited normal pain behaviour to noxious heat, suggesting that the role of TRPA1 may be exclusive to transduction of stimuli at low temperatures (Kwan et al. 2006).

1.2.5 The pain pathway

As described in section 1.2.3, primary afferent fibres enter the spinal cord and terminate in specific regions of the grey matter of the dorsal horn within the spinal cord (see (Millan 1999)). The cyto-architectural organization of the spinal cord was first described by the Swedish neuroscientist Rexed, based on the density and myelination of each of the 10

lamina (Rexed 1952). The primary afferents synapse onto second order neurones via chemical excitation by the neurotransmitter glutamate. The second order nociceptive dorsal horn inter-neurones appear as three main types; nociceptive specific which are mainly found in lamina I and respond to noxious stimuli only, non-nociceptive specific predominantly found in lamina II, III and IV and wide dynamic range neurones responding to a range of intensity of stimuli (Besson and Chaouch 1987; Dubner and Hargreaves 1989; Millan 1999). Axons from the wide dynamic range and nociceptive specific inter-neurones cross the spinal cord and typically ascend the spinothalamic tract conveying information to the thalamus. Other ascending pathways exist such as the spinoreticular, spinohypothalamic and spinocervicothalamic tracts projecting to different areas of the brain such as the mesencephalon and diencephalons (Besson 1999). Neurones within the thalamus project to the somatosensory cortex and anterior cingulate gyrus to allow the localisation and characterisation of painful stimuli and the perception and emotional response to the pain (see Willis and Coggeshall 1991).

1.3 Inflammatory joint pain

Inflammation is characterised by four common signs; the calor (heat), dolor (pain), rubor (redness), and tumor (swelling), caused by local vasodilation, oedema formation as inflammatory cells infiltrate the area and pain caused by chemical mediators causing nociceptive discharge. Pain arising from inflammation can be categorised into two different types as defined by the IASP committee on taxonomy: hyperalgesia is 'an increased response to a stimulus that is normally painful' and allodynia is 'pain due to a stimulus which does not normally provoke pain' (see Figure 1.4).

Primary hyperalgesia can exist at the immediate site of injury with secondary hyperalgesia in the tissue surrounding the damaged area. In the following sections I will describe the knee joint anatomy and summarise some of the important processes involved in

establishing inflammatory joint pain through mechanisms of peripheral and central sensitisation.

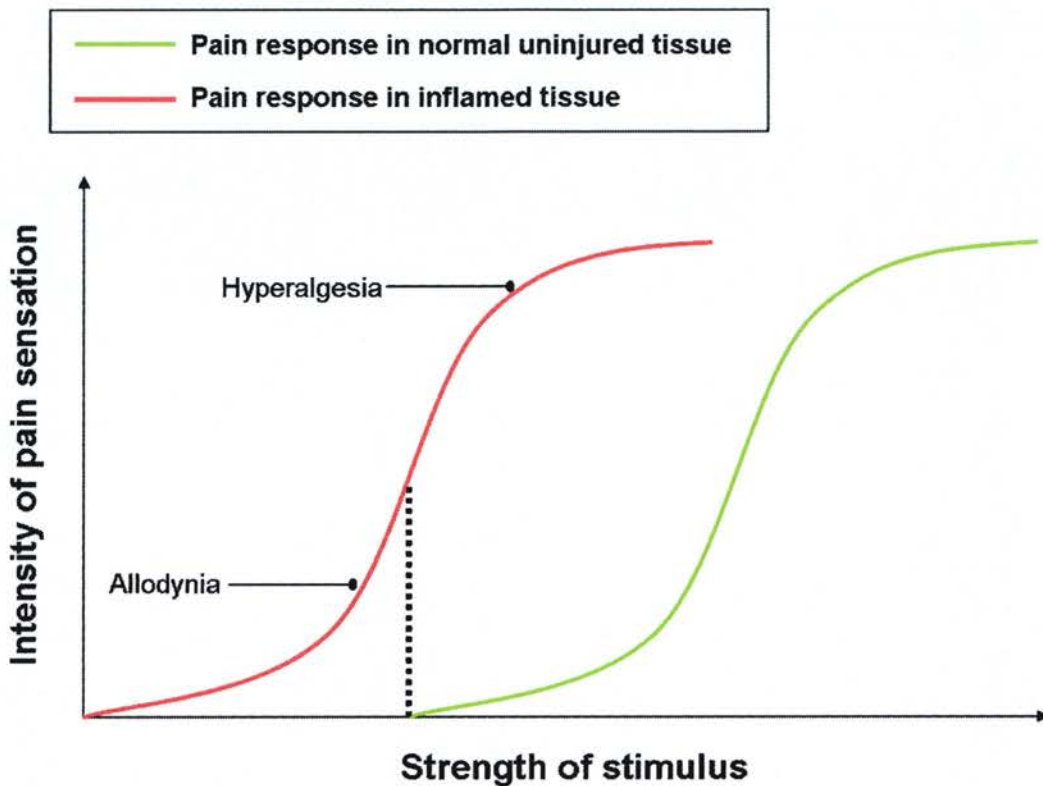


Figure 1.4 Sensitisation of pain responses from external stimuli. The normal response shown by the right hand curve (green) is shifted to the left following inflammation (red). A stimulus that is normally not noxious will now become so, a term described as allodynia, and a normally noxious stimulus will result in a higher intensity of pain sensation a term known as hyperalgesia. (illustration redrawn from (Gottschalk and Smith 2001).

1.3.1 Knee joint afferent nociceptors and inflammation

Anatomical studies of the feline knee joint provided the first information of the neurological innervation of the joint (Skoglund 1956; Freeman and Wyke 1967; Heppelmann et al. 1995; Heppelmann 1997). Further studies have described similar morphology in dogs (O'Connor 1984), rats (Hildebrand et al. 1991) and humans (Halata et al. 1985). Taken collectively, these studies show that there is very little difference in the neuronal architecture and types of fibres innervating the knee joint. The most notable inter-species difference was

the absolute number of afferents innervating the joint based on the overall size of the joint. Synovial joints are predominantly innervated by A δ - and C-fibres which terminate in non-corporcular endings with 60% of the afferents innervating the knee joint of the cat being classed as nociceptive (Heppelmann and Pawlak 1997). These afferents can be classified as belonging to one of five groups based on their sensitivity to pressure or movement within the joint (Coggeshall et al. 1983; Schaible and Schmidt 1983a; b; 1988):

- Group I : low threshold units that are weakly activated by innocuous pressure and movement in the joint
- Group II : high threshold units that are strongly activated by innocuous pressure and movement in the joint
- Group III : high threshold units that are only activated by noxious pressure and movement outside the normal range of the joint
- Group IV : units that respond to intense pressure but not movement
- Group V : silent nociceptors

The knee joint of the rat has three primary afferent nerves, namely the medial (MAN), lateral (LAN) and posterior articular nerves (PAN). The MAN nerve branches from the saphenous nerve and transverses the antro-medial aspect of the thigh running alongside the descending genicular artery and vein to the medial aspect of the knee (Freeman and Wyke 1967). Here the nerve splits into two branches and spreads to innervate various structures in the knee including: the ligamentum patellae, the fibrous capsule of the knee joint and the medial collateral ligament. The MAN of the cat contains approximately 630 afferents of which the majority (20% and 70% respectively) fall into either group III or group IV as described above (Langford and Schmidt 1983). Electrophysiological recordings made during this thesis were obtained from the MAN in rats. The rat MAN comprises approximately 400 axons innervating the rat knee joint (Hildebrand et al. 1991), with the

majority of the cell bodies of these sensory neurones being located in dorsal root ganglia (DRG) L3-L5 (Salo and Theriault 1997).

Peripheral sensitisation (described in section 1.3.2) during inflammation of the knee joint has been thoroughly characterised using electrophysiological studies, where pressure and movement of an inflamed joint lead to enhanced A β - and A δ -fibre responses (Schaible and Grubb 1993). In addition approximately 80% of A δ - and C-fibres in inflamed joints of cats are spontaneously active, firing at a rate of more than double in inflamed joints compared to normal joints (Coggeshall et al. 1983; Schaible and Schmidt 1985; 1988).

1.3.2 Peripheral sensitisation

Typically immediate pain results from tissue injury or damage, which causes the release of local inflammatory mediators from the damaged cells that act directly on terminals of peripheral nociceptors. Algogens such as 5-hydroxytryptamine (5-HT), K⁺, H⁺, ATP, nitric oxide, histamine, and bradykinin (BK) can all be released from damaged cells, leading to the classical inflammatory response, pain and the recruitment of cytokine releasing immune cells to the injury site (Kidd and Urban 2001). Some of these algogens depolarise the cell membrane immediately by acting through specific ligand-gated ion channels, for example 5-HT acting on the 5-HT₃ receptor, causes an immediate nociceptive neuronal discharge (Zemlan et al. 1988). Peripheral sensitisation occurs when these inflammatory mediators bind to G-protein coupled receptors and activate protein kinases (PK) which in turn phosphorylate the nociceptive receptors and ion channels, reducing the threshold required for nociceptive activation (Woolf and Salter 2000; Mackey 2004). Additionally, silent nociceptors that were previously inactive will be activated and add to the peripheral nociceptive drive, with the overall effect being an increased input to the spinal cord (Coggeshall et al. 1983; Schaible and Schmidt 1988; Schaible and Grubb 1993).

1.3.2.1 *Bradykinin (BK)*

BK is produced by enzyme mediated cleavage of kininogen found in the plasma at the site of tissue injury, and makes an early contribution to pain and inflammation through both a direct action on the nociceptors and secondary actions through indirect pathways (Dray et al. 1992; Dray and Perkins 1993). The direct action of BK is mediated through the B₂ receptors and causes depolarisation of the peripheral nerve terminals and propagation of an action potential (Beck and Handwerker 1974). Sensitisation of peripheral nerves by BK is mediated through the BK₁ receptor which is present in low numbers in normal tissues but enhanced expression is seen in inflamed tissues (Marceau et al. 1998). B₁ activation causes lowered threshold of activation and altered kinetics of VGSCs, reduced activity of voltage gated potassium channels (Wang et al. 2006), activation of the arachidonic acid pathway and increased sensitivity of primary afferents to heat stimuli (Lang et al. 1990). B₁ and B₂ null mutant mice demonstrated an attenuated acute inflammatory pain response to intra-plantar injection of adjuvant highlighting a role of the receptors in peripheral sensitisation (Fox et al. 2003). BK shows multiple complex interactions with other inflammatory mediators which lead to enhance peripheral sensitisation which are too numerous to discuss at this juncture. For a comprehensive review see (Perkins 1999).

1.3.2.2 *Eicosanoids*

COX-1 and COX-2 are induced in peripheral tissues following injury and inflammation (Dubois et al. 1998). The breakdown of arachidonic by the COX enzymes leads to the production and release of the prostaglandins and leukotrienes, collectively known as the eicosanoids, which act as sensitising agents. Such sensitising affects are mediated through the prostanoid receptor activating PKA which leads to a reduction in the activation threshold of VGSCs (England et al. 1996) and enhancement of the action of BK (Grubb et al. 1991). Although the eicosanoids are predominantly thought to act as sensitising

agents, PGE₂ and PGI₂ have been shown to directly excite afferent nociceptors through the EP₁₋₄ receptors (Birrell et al. 1991).

1.3.2.3 TRPV1

The TRPV1 receptor plays an integral role in the transduction of noxious thermal stimuli into nociceptive neuronal signals as described in section 1.2.4.2. In rats approximately half of the peptidergic and half of the IB4-positive primary afferent nerves express TRPV1 (Guo et al. 1999; Michael and Priestley 1999). TRPV1 receptors have been implicated in playing an important role in primary sensitisation of nociceptors, as during a model of peripheral inflammation, transport of the receptor mRNA to the periphery was increased and the proportion of TRPV1-labelled unmyelinated C-fibres was almost doubled (Carlton and Coggeshall 2001). Additionally, a similar model using carrageenan induced inflammation of the footpad in rats, showed the sensitivity of central terminals to capsaicin was increased, as evidenced by the increase in capsaicin-evoked release of glutamate from the dorsal horn (Tohda et al. 2001). Local inflammation induced by FCA increased TRPV1 protein levels in small and medium diameter DRG neurones (Amaya et al. 2003). A role for TRPV1 in inflammatory pain sensations was confirmed by use of the receptor antagonist capsazepine which reversed FCA-induced mechanical hyperalgesia in the Guinea-pig (Walker et al. 2003). In contrast to these studies it has also been reported that TRPV1 expression is not significantly altered in the acute or chronic stages of antigen-induced arthritis in the rat (Bar et al. 2004), which suggests that various pre-clinical models of chronic inflammatory pain may have different underlying pathophysiologies determined by the mode of action of the inflammatory agent or the species studied. Genetically modified mice lacking TRPV1 display clear and robust deficits in response to noxious heat stimuli, measured at the level of the cultured sensory neuron, the primary afferent fibre, and the spinal cord dorsal horn (Caterina et al. 2000).

Capsaicin-based tests are regularly used in the clinic to quantify neuronal sensitivity in patients suffering with RA (Morris et al. 1997). In these tests capsaicin is applied to the forearm of the patient for 30 minutes leading to the development of a mechanical hypersensitivity, but in patients suffering from RA this area of hypersensitivity is substantially larger than in normal patients implying enhanced central mechanisms in this disease (see section 1.3.3). Administration of capsaicin into the rat knee joint leads to a short term degeneration of unmyelinated C-fibres with no positive staining for CGRP or SP being observed in the knee joint up to 7 days after injection (Ferrell et al. 1992; Mapp et al. 1996), and this may account for the desensitising properties of topically applied creams used in the treatment of pain.

1.3.2.4 Neurogenic inflammation

Neurogenic inflammation is a term used to describe the contribution of sensory nerves to the local inflammatory response (Jancso et al. 1967; Foreman 1987a; Just and Heppelmann 2002). Stimulation of peptidergic fibres causes the release of the neuropeptides such as neurokinin A and B, SP and CGRP, which have inflammatory roles (Foreman 1987b; Snijdelaar et al. 2000). These neuropeptides lead to an increase in blood flow, vessel permeability and plasma extravasation first described following the observation that SP increased the proliferation of T lymphocyte into areas of inflammation (Payan et al. 1983). Antidromic nerve stimulation can lead to the local axon reflex with nociceptors outside the immediate area of stimulation releasing neuropeptides (Ferrell and Russell 1986). The release of neuropeptides causes mast cell degranulation and an increased release or formation of histamine, cytokines and the eicosanoids which in turn contribute to peripheral sensitisation (Kidd et al. 1990). The neuropeptides also exhibit numerous actions on leukocytes and the prostanoids (for review see McGillis and Fernandez 1999).

1.3.2.5 Cytokines

Pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF α), interleukin (IL)-1 β , IL-6 and IL-8, can be released from mast cells, leukocytes and synoviocytes and have been shown directly to sensitise nociceptors during inflammation (Bianchi et al. 1998; Dayer 2002; De Jongh et al. 2003). These cytokines cause the production and release of prostaglandins from surrounding cells (Dayer et al. 1986), and upregulate the levels of SP in peptidergic neurones (Jonakait et al. 1990). Furthermore cytokines regulate the increase in inflammatory cell recruitment, adhesion and activation, and both IL-1 and TNF α have been shown to play a pivotal role in the tissue destruction seen in chronic inflammatory diseases such as rheumatoid arthritis (Dayer 2002). An increase in cytokine levels within the rat knee joint following chronic inflammation has recently been described (Barton et al. 2007a; b).

Crucially all of these inflammatory mediators act to modify the normal response of afferent nerve fibres by reducing their activation threshold and increasing their excitability (Heppelmann and Pawlak 1997). In addition to inflammatory algogens causing peripheral afferent nociceptor sensitisation, up-regulation of receptors and ion channels such as TRPV1, the VGSCs, ASICs, opioid receptors and P2X / P2Y receptors have been associated with neuronal hyper-responsiveness. Of particular interest in this thesis are the opioid receptors and VGSCs which will be discussed in sections 1.5-1.7.

1.3.3 Central sensitisation

Central sensitisation, in the spinal cord and brain, occurs as a consequence of sustained or repeated activation of sensory afferent fibres terminating in laminae I, II and V of the dorsal horn (Woolf 1983; Bolay and Moskowitz 2002). Glutamate released from the terminals of these afferents normally acts on postsynaptic AMPA receptors to evoke a fast excitatory potential leading to depolarisation at the spinal level (Mendell 1966). Under the

physiological condition of inflammation, the release of neuropeptides, such as substance P and CGRP, leads to both AMPA and NMDA receptors becoming active in the processing of spinal neural signals and a long term increase in the excitability of the ascending dorsal horn neurones. NMDA receptors have been shown to play a key role in the development of central sensitisation in rats as a result of inflammatory pain (Sluka et al. 1994; Zhang et al. 2003). The overall result is that responsiveness of these postsynaptic cells to existing inputs and normally sub-threshold stimuli is increased and the receptive field size is expanded. This central sensitisation is responsible for the amplification of peripheral inputs from regions beyond the inflamed or injured tissue, a phenomena described in both humans and animals as secondary hyperalgesia (Schaible and Grubb 1993; Woolf and Salter 2000). Central sensitisation serves a protective purpose by making normally innocuous stimuli evoke pain responses in areas other than the initial site of injury to spare body parts from further injury whilst healing occurs (Woolf and Walters 1991). Administration of a local anaesthetic before pro-inflammatory agents such as capsaicin, blocks C-fibre activation and prevents central sensitisation in both rodents (Fitzgerald and Woolf 1982) and humans (LaMotte et al. 1992), proving that the process occurs as a result of repeated primary afferent stimulation. Central sensitisation can be studied in animals through the phenomenon known as wind up. Wind up is a frequency-dependent increase in the excitability of spinal cord caused by electrical stimulation of nociceptive C-fibres (Herrero et al. 2000). Wind-up is very useful in studying spinal cord physiology in rats, but it is not a process identical to central sensitisation, as it probably shares only a small component of the processes involved in central sensitisation as a result of peripheral inflammation (Herrero et al. 2000).

The experiments conducted in this thesis are focussed on the mechanisms involved in peripheral sensitisation during inflammatory joint pain, and although central sensitisation is an extremely important process in the overall perception of chronic pain, at this stage the mechanisms involved will not be explored any further.

1.4 Animal models of inflammatory pain

Several animal models of inflammatory pain exist that are commonly used to further the understanding of the underlying mechanisms involved in both acute and chronic pain states. These models are commonly used as pre-clinical screens for assessment of the efficacy of potential novel analgesics in the treatment of inflammatory pain. Generally these models rely on inducing agents to cause an inflammatory response and associated hypersensitivity (Le Bars et al. 2001).

1.4.1 Adjuvant-induced arthritis

Freund's complete adjuvant is an inflammatory agent first formulated by the Hungarian born American immunologist Jules Freund after whom the adjuvant was named (Freund and Lipton 1955; Freund 1956). FCA is a mixture of mineral oils, inactivated and heat killed *Mycobacterium tuberculosis* and emulsifying agents. Intradermal injection of FCA into the tail base of rats induces a polyarthritis (Pearson 1956), with the subsequent development of joint lesions nearly identical to those seen in human RA (Pearson 1963). The initial inflammation at the site of injection is followed by severe swelling in multiple joints approximately 5 days after FCA treatment (Pearson 1956; Glenn and Gray 1965; Pircio et al. 1975). It is thought that the mineral oil along with the peptidoglycans of the cell walls of the gram positive bacteria *Mycobacterium tuberculosis* that are present in FCA contribute to the immunostimulation within the animal, which leads to the development of arthritis (Whitehouse et al. 1974; Koga et al. 1976). Heat shock proteins have been implicated in the pathogenesis of human arthritis and a 65kD heat shock protein present in FCA contains a specific epitope corresponding to an amino acid sequence recognised by rat T cells (van Eden et al. 1988).

FCA-induced polyarthritis was soon suggested as being a suitable experimental model of chronic pain (De Castro Costa et al. 1981) as well as being used in studies of anti-

arthritic drugs (Rainsford 1982). Behaviours such as chronic scratching (Calvino et al. 1987), a reduced threshold to noxious heat and pressure (Millan et al. 1987) and hyperventilation (Colpaert 1987) were all observed in rats, validating this as a suitable model of chronic pain. The induction of polyarthritis using FCA is however associated with systemic disease and many undesirable side-effects such as severe weight loss, nodular lesions of the ears, feet, tail and genitals and diarrhoea (Pearson 1956). These side effects made it difficult to attribute the pain and hypersensitivity observed purely to joint arthritis. Furthermore as the welfare of laboratory animals is of highest priority, especially during chronic studies, the question of whether or not this model is ethically acceptable was raised (Casey and Dubner 1989).

Refinement of the polyarthritic model saw the development of a technique where FCA was injected sub-dermally around the tibio-tarsal joint of the ankle (Grubb et al. 1988; Donaldson et al. 1993). Further adaptation saw the FCA being injected directly into the articular space within the ankle joint of the rat (Butler et al. 1992). Both methods produce a stable monoarthritis lasting up to 30 days post-injection and beyond, with the FCA causing a joint hypersensitivity and similar pathology as seen in human RA. The animals did not display the irritability often observed in the polyarthritic model and gained weight in a normal fashion, an indication of normal feeding behaviour. This unilateral model of articular FCA administration has been applied to the knee joint of rats (Donaldson et al. 1993; Wilson et al. 2006) and mice (Chillingworth and Donaldson 2003; Gauldie et al. 2004) where a unilateral arthritis and associated joint hypersensitivity are induced without any systemic side effects. Interestingly, an accidental self injection of FCA into the forearm of a research worker has been documented to have resulted in severe inflammation that was successfully treated with steroids (Shah 2002).

For the work reported in this thesis FCA was injected into the articular space of the knee joint and was the method used to investigate peripheral sensitisation of nociceptive afferents during a model of chronic inflammatory joint pain. The model has already been established and characterized in this laboratory, meaning validation of the model was not

necessary for this thesis (Dowd et al. 1998a; b; Gauldie et al. 2001). Results were directly compared with these previous studies to ensure that similar outcomes were being observed. In this way the number of animals required for the studies was reduced.

1.4.2 Other models of inflammatory pain

A number of different inflammatory agents have been used in order to investigate acute inflammatory hypersensitivity in the hind paws of rats. Mustard oil applied topically on the foot pad causes vasodilation and formation of an oedema with an associated short lasting hypersensitivity due to the release of pro-inflammatory mediators from capsaicin-sensitive sensory neurones (Jancso et al. 1967). Substance P injected into the plantar region of the hind paw induces a dose related inflammation reaching a peak at 10 minutes and declining after 60 minutes (De and Ghosh 1990). Similarly an intraplantar injection of capsaicin causes pain-related behaviours such as guarding of the limb, licking of the paw and an unwillingness to apply pressure to the foot, and an associated hypersensitivity to various mechanical and thermal stimuli (Szolcsanyi et al. 1988; Gilchrist et al. 1996). Formalin and carrageenan are also commonly used as inflammatory mediators in studies investigating the mechanisms of action involved in acute inflammatory pain (Dubuisson and Dennis 1977; Bland-Ward and Humphrey 1997; Guay et al. 2004).

Animal models similar in principle to the FCA-induced unilateral arthritis have been used to produce a more persistent pain which has been likened to chronic pain observed in human joint disease. The use of sodium iodoacetate as a blocker of the glycolytic energy metabolism and synthetic processes in articular chondrocytes was first described over 20 years ago and produced osteoarthritic type reactions 2-4 months after a local injection in rats (Kalbhen 1987). More recently intra articular injection of sodium iodoacetate into the knee joint of rats has been validated as a model of OA, sharing many pathological changes that are observed in human OA and a chronic degeneration of the joint (Kobayashi et al. 2003). The

collagen induced arthritis model can be used in both mice and rats and provides an excellent animal model of RA, as it shares both immunological and pathological features seen in the human condition (Griffiths et al. 2007; Williams 2007).

1.5 Opioids

1.5.1 Opium alkaloids and morphine

Opium has been used in medicine for centuries and is obtained from the dried powdered juice of the poppy plant, *Papaver somnifrum* containing over 25 alkaloids such as morphine, codeine, thebaine and papaverine, which constitute its pharmacological activity. The average quantities of the alkaloids present in opium vary dependent on the sample, but the most abundant alkaloid is morphine, making up approximately 10% of the opium (Reynolds and Randall 1957). The structure of morphine was first established following its chemical synthesis in the mid-1900's (Gates and Tschudi 1952), although it was Sertürner in 1803 who first isolated the drug and gave morphine its name, a derivative of the Greek god of dreams, Morpheus. The pain relieving effects of the opioids, in particular morphine has been exploited for thousands of years, although the associated side effects such as sedation and nausea, together with the potential for abuse limit the clinical use of the drug. Despite the potential for its illicit use, morphine and the opioids have remained in use for longer than any other drug in history indicating its power as a strong analgesic, with the side effects often accepted (Schlug and Gandham 2006). To date morphine is still the gold standard opioid drug used in the treatment of severe pain.

1.5.2 The opioid receptor family

Binding assay work in the early 1970's using the opiate antagonist naloxone demonstrated the existence of specific opioid receptors in the brains of mice, rats, monkeys

and humans, with the distribution of the opiate-binding site being non-uniform (Kuhar et al. 1973; Pert and Snyder 1973; Simon 1973; Terenius 1973). Several classes of opioid receptor were revealed through the use of novel alkaloids which were being synthesised by chemists at the time (Martin et al. 1976). These results suggested the existence of three types of receptor which were named mu, sigma and kappa after the drugs used in the studies. It is now known that sigma is not an opioid receptor (Brownstein 1993) and has been replaced by the delta opioid receptor which was described later (Lord et al. 1977). To date three major opioid receptors, namely mu (μ), delta (δ) and kappa (κ), have been described from pharmacological studies, with the closely related opioid like receptor (ORL₁) receptor also mediating opioid like signals (Taylor and Dickenson 1998). Multiple μ (Pasternak 1993), κ (Traynor 1989) and δ (Traynor and Elliott 1993) receptor types have been proposed to exist as potential splicing of the three receptor genes could lead to variants in the receptors.

The discovery of these receptors prompted the search for endogenous ligands acting at any of these receptors. The pentapeptides met- and leu- enkephalin were the first endogenous opioids to be purified and sequenced (Hughes et al. 1975; Kosterlitz and Hughes 1975). Many more peptides sharing the opioid N-terminal sequence YGGFL/M have been purified from nervous tissue and the pituitary gland (for review see Akil et al. 1984). The enkephalins, dynorphins and β -endorphin are the three well defined endogenous families of opioid peptides. These peptides are produced in mammals from larger precursor protein named proenkephalin, prodynorphin and proopiomelanocortin respectively (Nakanishi et al. 1979; Kakidani et al. 1982; Noda et al. 1982a; b). Opioid peptides are widely distributed in the brain, with dynorphins and enkephalins occurring in the interneurons and descending pathways from the brain to the dorsal horn in the spinal cord respectively. These peptides are also produced by non-neuronal endocrine and exocrine glands such as the adrenal and pituitary gland, and in cells of the immune system (Stein et al. 1990a; Przewlocki et al. 1992). The three genes encoding these proteins were cloned in the early 1980's with each precursor giving rise to more than 20 opioid ligands (Takahashi et al. 1981; Comb et al.

1982; Kakidani et al. 1982). These opioid ligands act as agonists on the μ , δ and κ opioid receptors with nanomolar affinities and preferential selectivity (Akil et al. 1998).

Opioid receptors belong to the G-protein coupled family being coupled to the inhibitory G_i protein (Law et al. 2000) and through the inhibition of adenylate cyclase activate inwardly rectifying potassium channels and inhibit voltage gated calcium channels and hence decrease neuronal excitability (Burns et al. 1983). Traditionally the analgesic effect of opioids was thought to be solely attributed to receptors present in the central nervous system (Dubner and Bennett 1983; Basbaum and Fields 1984) with receptors being expressed throughout the brain (Bodnar and Klein 2005). More recently opioid receptors have been identified on peripheral nerve terminals in both rats and humans suggesting systemically administered opioids can provide analgesia through peripheral opioid receptor mediated effects (Stein 1995; Stein et al. 1996a). Immunostaining of small-diameter unmyelinated fibres in subcutaneous paw tissue of the rat first revealed opioid receptors were present in the periphery (Stein et al. 1990b).

1.5.3 Endomorphins

A new class of endogenous opioid peptides was recently discovered and isolated from bovine brain tissue (Zadina et al. 1997). Endomorphin-1 and -2 (EM1 and EM2) are tetrapeptides which show the highest affinity and selectivity for the μ -opioid receptor of any mammalian peptide studied to date and have now been isolated from the human cortex showing potent μ -opioid receptor bioactivity including analgesia (Hackler et al. 1997; Stone et al. 1997). EM1 is 4000- and 15000-fold selective and EM2 is 13000- and 7500-fold selective for μ -opioid receptors compared to δ and κ , respectively. EM1 is widely and densely distributed throughout the brain in areas such as the parabrachial nucleus and the nucleus of the solitary tract known to contain high densities of the μ -opioid receptor (Martin-Schild et al. 1999; Zadina et al. 1999). EM2 immunoreactivity is present in the amygdala,

hypothalamus, lower brain stem and the terminal regions of the spinal cord (Martin-Schild et al. 1997; Schreff et al. 1998; Wang et al. 2002; Wang et al. 2003) and in the capsaicin sensitive, peptidergic C-fibre primary afferent fibres (Martin-Schild et al. 1998). Both EM1 and EM2 inhibited C-fibre evoked responses of dorsal horn neurones in a dose dependent manner (Chapman et al. 1997) and show behavioural analgesic potency similar to morphine (Stone et al. 1997; Goldberg et al. 1998).

As well as mediating an analgesic effect, EM1 and EM2 act through the μ -opioid receptor to cause a decrease in heart rate, cardiac output and total peripheral resistance in the rat, leading to a decrease in systemic arterial blood pressure (Wood and Traynor 1989; Champion et al. 1997a; Czapla et al. 1998). Vasodilation in the rat hindquarters and a decreased blood flow in the knee joint suggest that EM1 and EM2 play an active role in the periphery (Champion et al. 1997b). Both EM1 and EM2 have been identified in the rat and human spleen (Jessop et al. 2000) and more recently macrophages and B cells (Seale et al. 2004). Evidence suggests that the endomorphins may play an integral role in controlling pain as a result of inflammation.

1.5.4 Opioid mediated analgesia

Clinicians are aware that patients display a wide range of responses when given opioids for the treatment of pain, and different doses are required to achieve the optimum response because individuals' sensitivity to the opioids is highly variable. Often in clinical practice a trial and error approach has to be adopted as in some cases patients will report one opioid is efficacious whereas another will provide no pain relief irrespective of the dose (Cherny et al. 2001). Suggestions as to why these variations exist include patients having genetic variation leading to differences in the availability of differently spliced opioid receptors and potential differences in pharmacogenetic factors regulating the transport of the receptors from the cell bodies to the target tissues (Kalso 2005). The prescribing of opioids

such as morphine and fentanyl is an established treatment route for patients with cancer and in palliative care settings (WHO, 1996). Guidelines for the use of such opioids in the treatment of pain in chronic non-cancer pain have been suggested with the aim of improving the patients' quality of life (Kalso et al. 2003). The appropriate and responsible prescribing of opioids has been the subject of considerable debate (Portenoy 1996b; Breivik 2003), with clinicians often keen to avoid the controversies and side effects associated with chronic opioid use such as addiction and tolerance (Portenoy 1996a; Breivik 2005). However, trials do exist reporting the long term safety and efficacy of taking opioids in randomised placebo-controlled trials. A systematic review in 2004 reported that the mean decrease in pain intensity in patients using opioids was 30%, yet only 44% were still using opioid therapy 7 to 24 months later, and just a minority of patients went on to use opioids as a form of long-term pain management (Kalso et al. 2004). Titration of remifentanyl (an ultra-short acting μ -agonist) against analgesia has been suggested as being a quick procedure to determine if a non-cancer patient is likely to respond to opioid treatment, and may provide a novel routine aiding the clinician in the decision of whether or not to prescribe opioids (Gustorff 2005).

1.5.4.1 Adverse side-effects of opioids

Physical and psychological dependence of opiates is displayed by a compulsive use of the drugs to the detriment of the user's physical and psychological health with hoarding and illegal acquisition of the drugs being common. In the clinic it has been suggested that addiction should be defined as a psychological and behavioural condition characterised by compulsive drug use and a loss of control, despite the drug causing more harm than good (Portenoy 1994). The increase in opioid drug abuse was highlighted by Gilson *et al.* in a 2004 review of five commonly prescribed opioids in which cases of abuse rose to 646% and 113% in the cases of fentanyl and morphine respectively (Gilson et al. 2004). Awareness and

guidelines in selecting patients to be treated with opioids are therefore very important (Portenoy 1996a; b).

Addictive drugs are thought to hijack the neural systems controlling the reward centres that evolved to mediate behaviours normally associated with food, water and sex (Hunt and Urch 2006). Three main theories for the reason of addiction have been proposed; a failure of the body to return to homeostasis following prolonged exposure and stimulation (Koob and Le Moal 1997), behavioural sensitisation leading to a craving of the drug (Robinson and Berridge 2003) and aberrant memory and learning mechanisms coming into play that establish abnormal drug habits (Berke and Hyman 2000). The neurobiology of drug addiction is not thoroughly understood but is thought to involve the mesolimbic and dopaminergic pathways, with the amygdala, ventral striatum and ventral tegmental area of the brain all being important.

Tolerance to opioids is seen in animals and humans (Collett 1998). The development of a tolerance to some of the side-effects of opioids such as nausea and vomiting may occur, although it is never seen in regards to constipation. Tolerance to the analgesic component of opioids is far more controversial with the need to increase doses over time to achieve similar efficacy. Many patients treated for either cancer or non-cancer related pain can achieve a stable dose of a specific opioid without requiring an escalating need for the drug (Glynn and Mather 1982; Portenoy and Foley 1986), and only increase their dose in response to increased pain (Chapman and Hill 1989). For patients showing a disposition to tolerance, rotation of the opioid preparations taken could remove the need to raise the doses and lead to better management of a potential tolerance to the drugs (Fallon 1997; Twycross 1998).

As well as exhibiting properties of addiction and tolerance, opioids can produce multiple adverse side-effects such as nausea and vomiting, constipation, respiratory depression, sedation, hallucinations and pruritus (Schug et al. 1992; Lawlor and Bruera 1998). These effects are variable from patient to patient and are dependent on the route and speed of administration, progression and severity of the pain, interactions with other drugs

the patient is taking and the patient's emotional status. Of particular concern are respiratory depression and constipation. Respiratory depression is the most common cause of opioid related death and older patients and those with underlying respiratory disease are most at risk (Schug et al. 1992). Constipation is the most commonly occurring side effect and although not life threatening can be really uncomfortable. Activation of opioid receptors in the bowel leads to decreased peristalsis, increased sphincter muscle tone and decreased intestinal secretions. Extreme forms of constipation can present themselves in the form of narcotic bowel syndrome (Sandgren et al. 1984). Recent studies using peripherally restricted opioid antagonists have tried to eradicate this problem without compromising the centrally mediated analgesic effects (Choi and Billings 2002; Kurz and Sessler 2003).

At this stage it is important to note that the majority of these side effects appear to be centrally mediated and as the focus of this thesis is the peripheral mechanisms of opioids then they shall be discussed no further. A review of the neurobiology involved in these side effects can be found in Wall and Melzack's textbook of pain (Hunt and Urch 2006; Schug and Gandham 2006) and (Bodnar and Klein 2005).

1.5.5 Opioids and inflammation

1.5.5.1 Opioid receptors in the periphery

Up until the late 1970's the belief was that the antinociceptive effects of opiates operated solely through the activation of centrally located opioid receptors. These receptors are made in the DRG, and it is perhaps not surprising that they can be transported to the peripheral sensory nerves as well as central terminals. Multiple opioid receptors have been shown to exist on primary afferent nerves (Fields et al. 1980; Stein et al. 1990b; Hassan et al. 1993; Coggeshall et al. 1997). Various animal behavioural tests were performed throughout the 80's showing that peripherally-administered or restricted opioid antagonists could

attenuate pain related behaviours that were in turn antagonised by naloxone (for review see Stein 1991). Intra-plantar injection of μ , δ and κ receptor agonists produced an increase in the paw withdrawal threshold from a mechanical stimulus in an acute model of FCA-induced footpad hypersensitivity in rats, interestingly this increase was only observed in inflamed paws and not normal paws (Stein et al. 1989). Notably, opioid receptors have been shown to be functional in the knee joint of both rats and humans in inflammatory conditions (Stein et al. 1990a; Stein et al. 1996a).

1.5.5.2 Peripherally mediated analgesia in inflammatory joint pain

Evidence began to build that peripherally administered opioids produced analgesia in inflammatory conditions (Ferreira and Nakamura 1979; Joris et al. 1987; Stein et al. 1988a; b; c). In the clinic, patients suffering from inflammatory joint diseases such as OA and RA were receiving effective analgesia from morphine injected directly into the knee joint without suffering the centrally mediated side-effects. Moreover the analgesia lasted for weeks after a single injection (Stein et al. 1991; Joshi et al. 1993; Stein 1995; Likar et al. 1997; Stein et al. 1999).

Rat models of acute inflammation show that there is an enhanced axonal transport of the μ -opioid receptor from the DRG to the inflamed tissue (Hassan et al. 1993; Ji et al. 1995), resulting in an up-regulation of the receptor at peripheral nerve terminals (Stein et al. 1990b). Furthermore an up-regulation in the μ -opioid receptor mRNA present in DRG tissue following rat hind-paw inflammation can be seen (Zhang et al. 1998). A recent study suggests that μ -opioid receptors are in fact down-regulated at the site of inflammation (Li et al. 2005) as the receptors are being transported to the periphery and the receptor production in the DRG is not increased (Schafer et al. 1995). This appears to contradict both a previous chronic inflammation study in rats where the μ -opioid receptors were up-regulated following systemic FCA administration (Ballet et al. 2003), and the clinical efficacy of intra-articular

morphine in humans with RA, as described above. The effect of inflammation on the δ and κ opioid receptors is less well investigated, with some reports suggesting that the δ -opioid receptors are either down-regulated or appear to remain unchanged in DRG of rats following acute, adjuvant-induced inflammation (Ji et al. 1995; Maekawa et al. 1996). In contrast the δ -opioid receptor has been seen to be up-regulated in a chronic model of FCA-induced inflammation suggesting the changes in the expression of the opioid receptor are determined by the stage of inflammation, during the transition from acute to chronic inflammatory pain (Cahill et al. 2003). Several studies investigating peripherally restricted opioid agonists have revealed analgesic effects mediated through all three of the opioid receptors (DeHaven-Hudkins et al. 1999; Machelska et al. 1999; Wu et al. 2006).

1.5.5.3 Inflammatory cells

Following tissue injury or an inflammatory insult, immune cells such as the leukocytes infiltrate the tissue as described in section 1.3.2. These cells release endogenous opioids, such as β -endorphin, in response to IL-1 β and corticotrophin releasing hormone present at the site of inflammation (Cabot et al. 1997; Cabot et al. 2001). Small inactive cytokines, injected into the footpad of rats elicited opioid release even in the absence of injury or inflammation (Schafer et al. 1994; Schafer et al. 1996; Machelska and Stein 2002). It appears that intrinsic pain inhibition is proportional to the number of invading leukocytes (Rittner et al. 2001) and blocking the infiltration of these cells reduces the peripheral pain control (Machelska et al. 1998; Machelska et al. 2002). In rats it appears that in the first 48 hours post-inflammatory insult, the opioid mediated analgesia is critical on the recruitment of leukocytes via a cytokine mediated process and the presence of receptors at the site of injury (Brack et al. 2004a; b). With EM1 and EM2 recently being shown to appear in inflammatory cells (Jessop et al. 2000; Seale et al. 2004) it appears that the body has an

endogenous opioid mechanism for helping control pain at sites of inflammation and injury in the periphery.

1.6 Voltage gated sodium channels (VGSCs)

1.6.1 The sodium channel family

Thermal, mechanical and chemical stimuli are detected in the periphery by sensors and an excitatory action potential is transmitted to the dorsal horn of the spinal cord conveying the information. This process is mediated in part through voltage gated calcium and potassium channels, but mainly through VGSCs as they are responsible for the initial depolarisation of the membrane (Hodgkin and Katz 1949; Hodgkin and Huxley 1952a; c). To date, nine different sodium channel isoforms with corresponding genes have been discovered in mammals (Goldin et al. 2000). A nomenclature system based on that used for voltage gated potassium channels (Chandy 1991) has been adopted and is now the most commonly used. This system classifies the channels as $\text{Na}_v1.1$ through to $\text{Na}_v1.9$. All these channels have been identified as having greater than 50% homology in amino acid sequence (Goldin et al. 2000). Sodium channels comprise an alpha subunit and several supporting beta subunits. The alpha subunit consists of four homologous domains each containing six transmembrane segments and is approximately 2000 amino acids long (Catterall et al. 1986). The alpha subunit contains the pore forming structure, an ion selectivity filter, an inactivation loop and the voltage sensing machinery, thus a single alpha subunit constitutes a functional VGSC (Catterall 2000). The beta subunits target and anchor the alpha subunit to specific sites on the plasma membrane and modulate gating properties (Isom 2001).

The VGSCs have also been further sub-divided into two general groups one in which the channels are blocked by tetrodotoxin (TTX-sensitive, TTX-S) and the other where the channels are resistant to TTX (TTX-resistant, TTX-R). $\text{Na}_v1.1$, $\text{Na}_v1.2$, $\text{Na}_v1.3$, $\text{Na}_v1.4$,

Na_v1.6 and Na_v1.7 all generate the TTX-S component sodium current (Rush et al. 1998). These TTX-S channels are blocked by TTX in the nanomolar range, have low thresholds of activation and are both rapidly activated and then inactivated. The TTX-R channels Na_v1.5, Na_v1.8 and Na_v1.9 tend to have high thresholds of activation, that are slow to activate and then subsequently inactivate (see Blair and Bean 2002 for review). VGSCs are differentially expressed throughout various subpopulations of sensory neurones with Na_v1.6 and Na_v1.7 being present in virtually all sensory neurones in rats (Black et al. 1996). Na_v1.3 and Na_v1.5 are expressed at high levels in embryonic sensory neurones but very low levels in adult, suggesting these channels have an important developmental role (Waxman et al. 1994; Renganathan et al. 2002). Unmyelinated sensory C-fibres all express Na_v1.7, with Na_v1.9 being associated exclusively with these nociceptive neurones (Fang et al. 2002; Djouhri et al. 2003b). Both Na_v1.8 and Na_v1.9 are present in most but not all nociceptors (Fang et al. 2002; Djouhri et al. 2003a). During an FCA-induced rat model of inflammatory pain the sodium channels collectively are dramatically increased up to two months after subcutaneous injection of the adjuvant into the hind-paw (Gould et al. 1998).

1.6.2 Role of sodium channels in inflammatory joint pain

1.6.2.1 Na_v1.7

Recent clinical studies have provided convincing evidence that Na_v1.7 plays a crucial role in the detection of pain and pain sensitivity. Families suffering from congenital indifference to pain (CIP), a condition characterised by an absence in the ability to sense pain, all suffer from a mutation in the SCN9A gene that encodes Na_v1.7 that leads to a complete loss of functionality of the channel (Cox et al. 2006; Fertleman et al. 2006; Ahmad et al. 2007). Conversely paroxysmal extreme pain disorder (Fertleman et al. 2006) and inherited erythromelalgia (Lee et al. 2007) are two autosomal dominant pain conditions

where point mutations in the SCN9A gene cause hyperpolarising shifts in the voltage dependence of activation of the channel leading to increased neuronal excitability. These mutations cause severe rectal and facial pain, and chronic burning sensations in the hands and feet in the respective conditions.

An increase in $\text{Na}_v1.7$ mRNA and protein levels are observed as a result of exposure to nerve growth factor (Toledo-Aral et al. 1995) and carrageenan in rats (Tanaka et al. 1998; Black et al. 2004). Knockdown of the channel in a predominantly nociceptive population of mice DRG neurones reduces both acute and chronic pain behaviours (Nassar et al. 2004; Yeomans et al. 2005). Following an intra-plantar injection of FCA in rats, the labelling of $\text{Na}_v1.7$ in L4-S1 DRG neurones 24 hours post-FCA injection was significantly increased (Gould et al. 2004). A study evaluating the expression of the channel in painful human teeth revealed a dramatic increase in $\text{Na}_v1.7$, contributing to spontaneous pain and increased pain levels experienced in dental disease (Sorensen et al. 2003). Trafficking of these functional $\text{Na}_v1.7$ channels into the nociceptive membrane could account for a portion of the increased neuronal excitability following inflammatory injury (Wada et al. 2004).

1.6.2.2 $\text{Na}_v1.8$

The role of $\text{Na}_v1.8$ in chronic inflammatory pain has been extensively studied in rats with rapid upregulation commonly seen in the early stages of inflammation following various inflammatory insults such as carrageenan, (Black et al. 2004), FCA (Gould et al. 2004) and prostaglandin E_2 (Villarreal et al. 2005). In particular $\text{Na}_v1.8$ has been implicated in the maintenance of inflammatory pain as there is a significant increase in channel mRNA in the DRG tissue of male Sprague-Dawley rats, four days after carrageenan injection (Tanaka et al. 1998). The increase in channel expression is associated with re-distribution of the channels to peripheral nerve terminals as FCA-induced inflammation results in an increase in the amount of $\text{Na}_v1.8$ channels observed in peripheral axons (Coggeshall et al.

2004). $\text{Na}_V1.8$ knockdown mice have reduced pain behaviours following a peripheral inflammatory insult (Khasar et al. 1998; Joshi et al. 2006) and $\text{Na}_V1.8$ null mutant mice show a delayed hypersensitivity following inflammatory insult (Akopian et al. 1999). Modulation of this channel by inflammatory mediators has also been shown to increase the magnitude of the $\text{Na}_V1.8$ current by increasing activation and inactivation rates and thus increasing neuronal excitability (Gold et al. 1996). Examples of this modulation have been shown to be caused by protein kinase A (Fitzgerald et al. 1999), PGE_2 (England et al. 1996) and endothelin-1 (Zhou et al. 2002).

With the majority of studies showing elevated levels of $\text{Na}_V1.8$ that do not return to pre-insult levels, it has been speculated that the channel plays a key role in the maintenance seen in chronic inflammatory pain signalling in rodents. This makes the channel a desirable target for novel analgesics, as prophylactic administration of channel blockers preventing the establishment of inflammatory pain in the first place is an unachievable prospect. Intrathecal administration of the μO -conotoxin peptide MrVIB taken from cone snail attenuated the reduction in thermal and mechanical paw withdrawal thresholds following intra-plantar FCA injection in male rats (Ekberg et al. 2006). Interestingly, MrVIB was shown to protect rats against acute post incisional pain when injected into the footpad prior to making the incision suggesting the channels may also play an important role in the establishment of inflammatory pain (Bulaj et al. 2006). The molecule A-803467 is a potent and selective inhibitor of $\text{Na}_V1.8$ channels that showed significant efficacy in reducing both mechanical and thermal hypersensitivity in rats subjected to a range of acute inflammatory insults (Jarvis et al. 2007).

1.6.2.3 $\text{Na}_V1.9$

There are limited studies describing the role of the $\text{Na}_V1.9$ channel in inflammatory pain conditions, and those that do exist provide conflicting data as to whether the channel is

up-regulated, down regulated or unchanged during the typical inflammatory insults. In rats, inflammation induced an up-regulation in $\text{Na}_v1.9$ mRNA seven days after an FCA insult (Tate et al. 1998), whereas Black et al. (2004) report no change in the expression of $\text{Na}_v1.9$ mRNA and protein in DRG four days after an intra-plantar carrageenan injection. Furthermore a decrease in the expression of the channel on primary afferent nerves in rats two days after FCA-induced inflammation has been described (Coggeshall et al. 2004). $\text{Na}_v1.9$ null mutant mice have been developed that show reduced sensitivity to the inflammatory mediators FCA, prostaglandin E_2 and carrageenan (Amaya et al. 2006). Studies using these mice revealed that the channel contributes to the persistent thermal hypersensitivity and spontaneous pain behavior seen following peripheral inflammation (Priest et al. 2005).

1.7 Aims of the thesis

The primary aim of this thesis was to test the hypothesis that functional opioid receptors are present in the periphery during a model of FCA-induced joint inflammation, and to subsequently determine whether EM1 is an effective treatment for the chronic pain associated with the inflammation. A secondary aim, undertaken during my placement at GlaxoSmithKline, was to investigate how the expression of VGSCs change during the development and subsequent maintenance of chronic inflammatory joint pain, concentrating on $\text{Na}_v1.7$, $\text{Na}_v1.8$ and $\text{Na}_v1.9$, due to their high levels of expression on nociceptive afferents. In each case a combination of techniques including, *in vivo* electrophysiology, behavioural studies and immunohistochemistry were used to elucidate the role that the receptors and channels play in establishing a chronic joint hypersensitivity.

Additionally a new piece of laboratory equipment capable of measuring primary mechanical hypersensitivity of both the rat and mouse knee joint was developed and validated as a novel behavioural readout for assessing experimental joint pain.

Chapter 2

General Methods

2.1 Animal models of experimental arthritis

2.1.1 Animals

All experimental procedures described in this thesis were carried out in accordance with the regulations and guidelines of the scientific procedures act (1986). Work was performed under the personal licence PIL 60/9547 and the UK Home Office project licences PPL 80/02019 at GlaxoSmithKline and PPL 60/2750 (Nov 04 – Aug 05) and PPL 60/3496 (Aug 05 onwards) at the University of Edinburgh unless stated otherwise.

402 male Wistar rats (Charles River, UK) and 24 male C57 Black-6 mice (C57BL/6, Charles River, UK) were used in all the studies reported. Rats were housed in groups of a maximum of 6 animals and mice in groups of 4 under a 12-hour light/dark cycle with free access to water and standard animal feed. Animals were acclimatised for at least 5 days following arrival prior to commencing experimental studies.

Endeavours were made throughout all studies to fulfil the 3R Home Office guidelines of refining experimental design and techniques to minimise animal distress and reducing the total number of animals used.

2.1.2 FCA-induced unilateral arthritis of the rat knee joint

A unilateral knee joint arthritis was induced in the left (ipsilateral) knee joint of adult male Wistar rats (150-200g) by an intra-articular injection of Freund's complete adjuvant (FCA, 1mg.ml⁻¹ *Mycobacterium tuberculosis* suspended in heavy liquid paraffin oil with Monooleate adjuvant, Sigma, UK). All rats were transiently anaesthetised using 3% isoflurane in oxygen and had their left knee shaved and cleaned using a dilute Hibiscrub solution. Rats were then injected with 150µl of FCA into the knee joint space using a 29-gauge needle (BD Microfine, USA). The patella tendon was used to orientate the needle with the injection site. Animals were allowed to recover from anaesthesia before being returned to

their cage. Following FCA injection the animal's mobility and general behaviour was observed and body weights were recorded to ensure the general health of each animal was not compromised as a consequence of the injection. Rats receiving the injection of FCA are defined throughout this thesis as FCA-injected, with animals receiving no injection being termed naïve.

Trial injections using the dye Evans Blue (Sigma, UK) were performed on cadavers prior to any studies taking place to ensure 150µl of FCA could be injected into the knee joint cavity without visible leakage.

2.1.3 FCA-induced unilateral arthritis of the mouse knee joint

A unilateral knee joint arthritis was induced in the left (ipsilateral) joint of adult male C57BL6 mice (23-28g) by an intra-articular injection of FCA (1mg.ml⁻¹, *Mycobacterium tuberculosis*, Sigma, UK). Mice were transiently anaesthetised using 3% isoflurane in oxygen and a small incision was made over the left knee joint which enabled the patella tendon to be visualised. Mice were then injected with 20µl of FCA directly into the joint space of the knee joint with the 30-gauge needle (BD Microfine, USA) mounted on a 50µl Hamilton syringe passing through the patella tendon. Naive animals were untreated. Animals were allowed to recover from anaesthesia before being returned to their cage. Following FCA injection the animals' mobility and general behaviour was observed by the licensee and the named animal care welfare officer and body weights were recorded to ensure the general health of each animal was not compromised as a consequence of the injection.

2.2 Behavioural assessment of arthritis

2.2.1 Assessment of weight distribution

An evaluation of how the animals distribute their weight across the hind limbs when standing was measured using either a rat or mouse incapacitance tester (Linton Instruments, UK). The apparatus comprises two force transducers mounted side by side, capable of measuring the downward force placed through them over a set period of seconds (see Figure 2.1). Animals were placed in a Perspex holding box with each hind limb centred on a transducer platform. The force placed through each transducer was recorded over a 3 second period (Clayton et al. 1997). The 'weight' carried by the FCA-injected ipsilateral limb was divided by that carried by the un-injected contralateral limb to provide a weight distribution (WD) ratio. A ratio of 1.0 resulted when the animal distributed its weight equally across both hind limbs, as occurs in naïve animals.

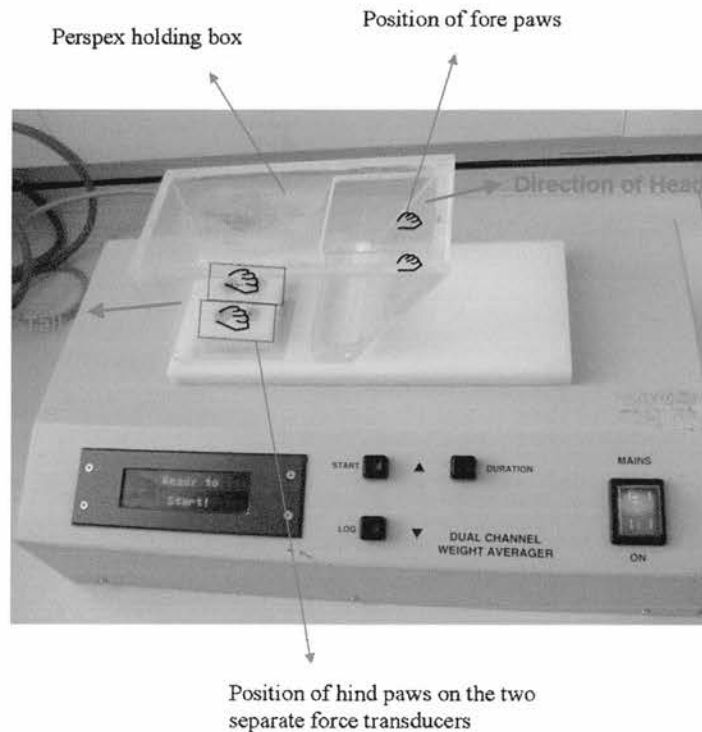


Figure 2.1 Photograph of the rat incapacitance tester used to assess the weight distribution of rats. An identical device but with a smaller Perspex holding box and smaller force transducers was used for measurements in murine studies.

2.2.2 Assessment of limb withdrawal threshold

Animals were lightly but securely held by a colleague and a gradually increasing squeeze was applied across the knee joint between the thumb unit and forefinger of the operator. At the point the force applied across the knee became noxious, the animal withdrew its leg from the stimulus and this was the test endpoint. Very rarely the animal vocalised before withdrawing from the apparatus and on these occasions vocalisation was taken as the test endpoint. The maximum force (gf) applied across the joint was automatically recorded on the digital display and noted as the limb withdrawal threshold (LWT). Ipsilateral and contralateral LWTs were taken for each animal with the ipsilateral limb being measured first and the contralateral limb acting as the internal control. The FCA-injected ipsilateral LWT was divided by the un-injected contralateral LWT to give a PAM withdrawal threshold ratio.

The full details of the development and validation of the PAM device for use in both rats and mice is covered in Chapter 5 of this thesis.

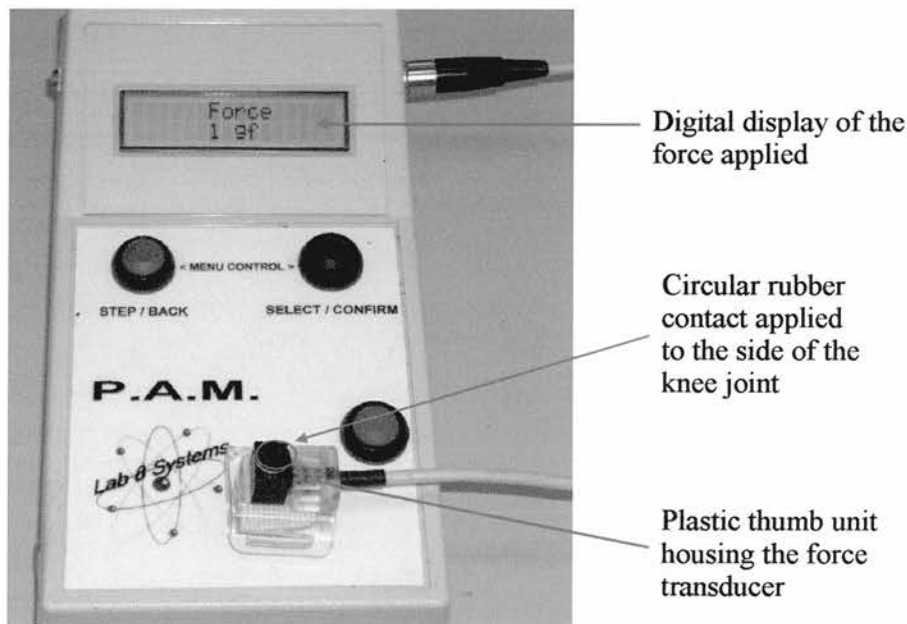


Figure 2.2 Photograph of the PAM device used to assess the limb withdrawal threshold of rats when a localised pressure was manually applied across the knee joint. The same base unit was used but with a smaller (in diameter) force transducer connected when assessing the limb withdrawal threshold of mice.

2.2.3 Assessment of inflammation induced by FCA

A single measurement of the joint diameter of both the ipsilateral and contralateral knee was measured before and then at 24 hours and subsequently on selected days post-FCA injection using a hand held digital micrometer (Mitutoyo, Japan; see Figure 2.3). The diameter of the ipsilateral joint was expressed as a ratio of the contralateral joint diameter.



Figure 2.3 Photograph of the digital micrometer used to measure the knee joint diameter of both rats and mice.

2.3 Recording electrical activity from the primary afferent neurons innervating the knee joint

2.3.1 Anaesthesia and general surgical procedures

Extracellular neuronal recordings were made *in vivo* from the MAN of the left knee joint in both naïve and FCA-injected rats. Initially rats were anaesthetised with pentobarbital sodium (60mg.ml⁻¹, i.p.). The animal was laid on its back on a heating blanket and a thermistor probe was inserted into the rectum. The heating blanket was homeostatically controlled to maintain the animal's core body temperature at 37°C (Harvard Apparatus Limited, UK). Before any surgical procedures began, and at regular intervals throughout surgery, forceps were used to pinch the footpad of the hind-paw to ensure the animal was at a suitable level of anaesthesia, determined by the lack of a reflex limb withdrawal.

Once fully anaesthetised animals had their trachea intubated to facilitate breathing and allow artificial ventilation (Ventilator, 50-1718, Harvard Apparatus Limited, UK) if required (see Figure 2.4). In order to monitor the arterial blood pressure (B.P.), the right carotid artery was cannulated (outer diameter of cannula = 0.75mm) and linked to a Maclab/4S (AD Instruments, UK) through a pressure transducer (Bell and Howell, UK) so a live B.P. trace was displayed and recorded by the program Chart v.5 using an Apple Mac.

The right femoral artery and vein were cannulated to allow test drugs and anaesthetic to be administered throughout the experiment. The arterial cannula (outer diameter of cannula = 0.75mm) was inserted into the lower part of the descending aorta, above the femoral bifurcation so that following intra-arterial (i.a.) drug delivery normal blood flow would carry the drug to the left hind-limb. The right femoral vein was cannulated (outer diameter of the cannula = 0.63mm) to allow a continuous infusion of pentobarbital ($325\mu\text{l}.\text{hr}^{-1}$) diluted to 40% in saline ($24\text{mg}.\text{ml}^{-1}$). The B.P. was recorded for the duration of each experiment and was ideally maintained at around 130/90mmHg. If the systolic blood pressure dropped below 80mmHg and could not be recovered by turning off the infusion of anaesthetic then the experiment was not analysed beyond that point.

2.3.2 Peripheral nerve dissection – the medial articular nerve (MAN)

Once the general surgical procedures had been performed and blood pressure and anaesthesia was stable, the peripheral nerve was dissected. To achieve this, the left hind limb was fixed in plaster of Paris to a brass support plate held fast in a stand and clamp arrangement. An incision was made in the skin around the medial aspect of the knee to produce a circular well of approximately 25mm in diameter where the MAN could be seen to branch at 90° towards the knee away from the saphenous nerve. A circular well was formed using thread to stitch the exposed edges of the skin to a brass ring held on the stand and clamp. The pouch was filled with heavy liquid paraffin (HLP, Sigma UK) oil to create an

electrically isolated system (see Figure 2.4). The saphenous nerve was cut proximally to eliminate any descending efferent neural activity from the recording and input from skin afferents was removed by separating the skin and overlaying connective tissue from the tissue surrounding the knee joint using blunt forceps. The MAN was carefully dissected out from the surrounding tissue using fine forceps and was cleaned and the whole nerve was placed over the platinum/iridium wire electrodes (Pt90/Ir10, 0.125mm, Goodfellow, UK) soldered to a bipolar recording pen.

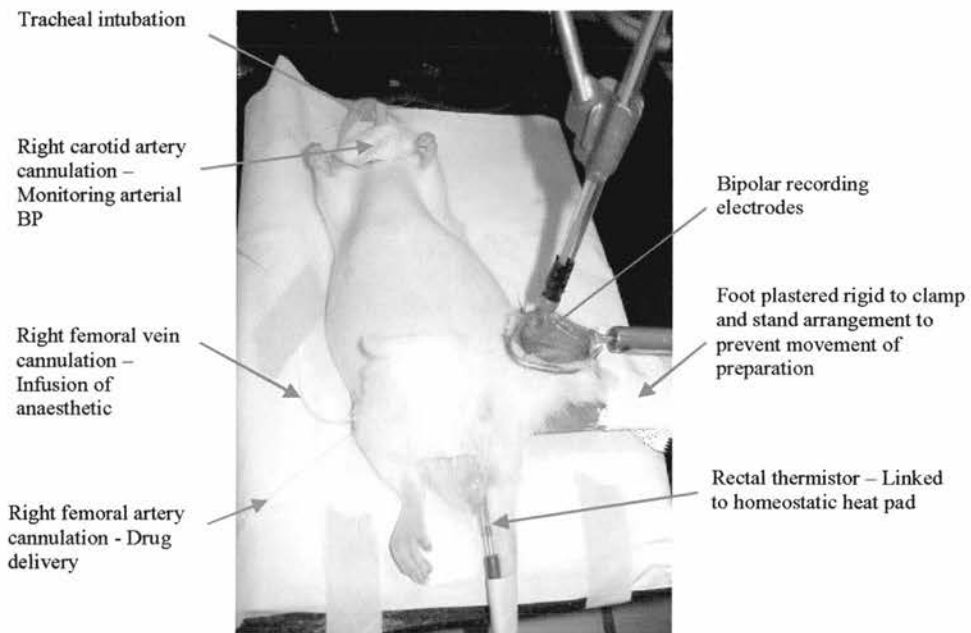


Figure 2.4 Photograph of an anaesthetised rat in a typical experimental setup for recording the neuronal activity of the afferents of the MAN innervating the rat knee joint. The left hind-paw was fixed to a solid brass platform using plaster of Paris to enable a stable recording to be taken.

2.3.3 Single electrode recording from the MAN

The afferent activity of the whole MAN was recorded as the potential difference between the two platinum/iridium wire electrodes across which the nerve was placed. The raw electrical signal was passed from the electrode into the head-stage (Digitmer Ltd., UK) and then onto an AC pre-amplifier (Neurolog NL104, Gain = 20,000, Low Pass = 0.1Hz). The signal was filtered (10Hz - 1000Hz bandwidth; Neurolog, NL115), displayed on an

oscilloscope (DL1540C, YokoGawa, Japan) and connected to a loud speaker (audio amp, Neurolog NL120). Finally the neural signal was digitally processed by a Micro 1401 MKII interface (Cambridge Electronic Design, UK) connected to a personal computer running Spike 2 software (CED, UK; version 5.01, see Figure 2.5 for summary of the signal processing). Events or tests were marked on the recording as text on the trace, and drug delivery and test points were noted using these keyboard markers, as well as in an experimental laboratory book. Data files were saved and later analysed offline following the completion of the experiment. A typical *in vivo* electrophysiology recording was conducted for between four and six hours.

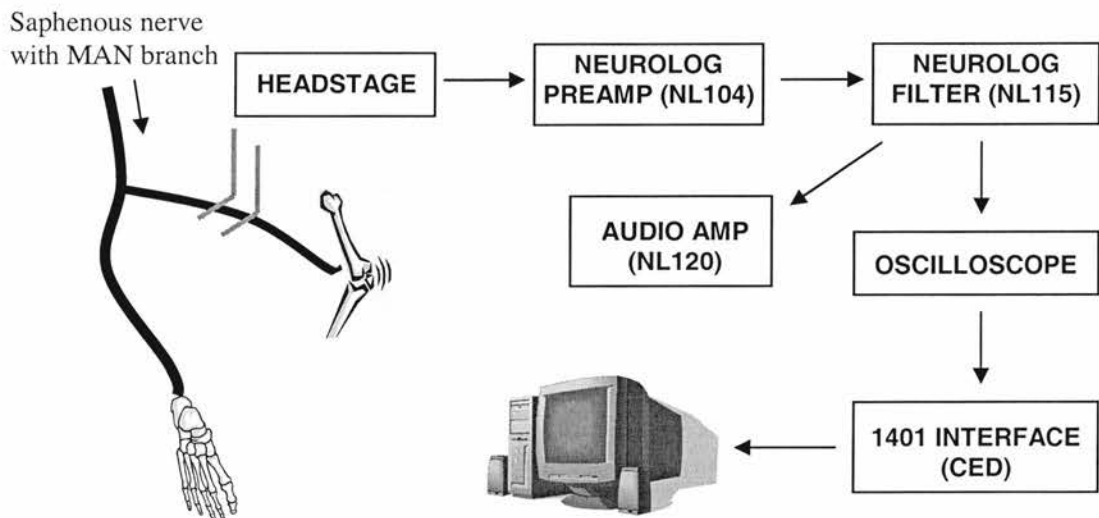


Figure 2.5 A schematic diagram showing how the raw neuronal signal from the MAN was collected, processed, digitised and then stored on a PC.

2.3.4 Spontaneous neural activity

The spontaneous activity of the afferents in the MAN bundle was measured in the first instance during the first 600s of each experiment, and for a period of 10s before any mechanical stimulation or drug administration (see Figure 2.6). Spontaneous activity was measured as both the absolute number of action potentials (A.P.) and the action potential frequency (impulses. s^{-1}) recorded during the test period.

2.3.5 Drug administration

Drugs were administered close to the knee joint in a volume of 100 μ l via the cannula in the right femoral artery and immediately flushed with 200 μ l of saline. The latency (sec) from the drug injection to the commencement of neuronal discharge and the duration (sec) of the firing were both measured. The effect of the drug was expressed as the change in either the absolute number of A.P. or the action potential frequency (impulses.s⁻¹), between that of the test period and the spontaneous period.

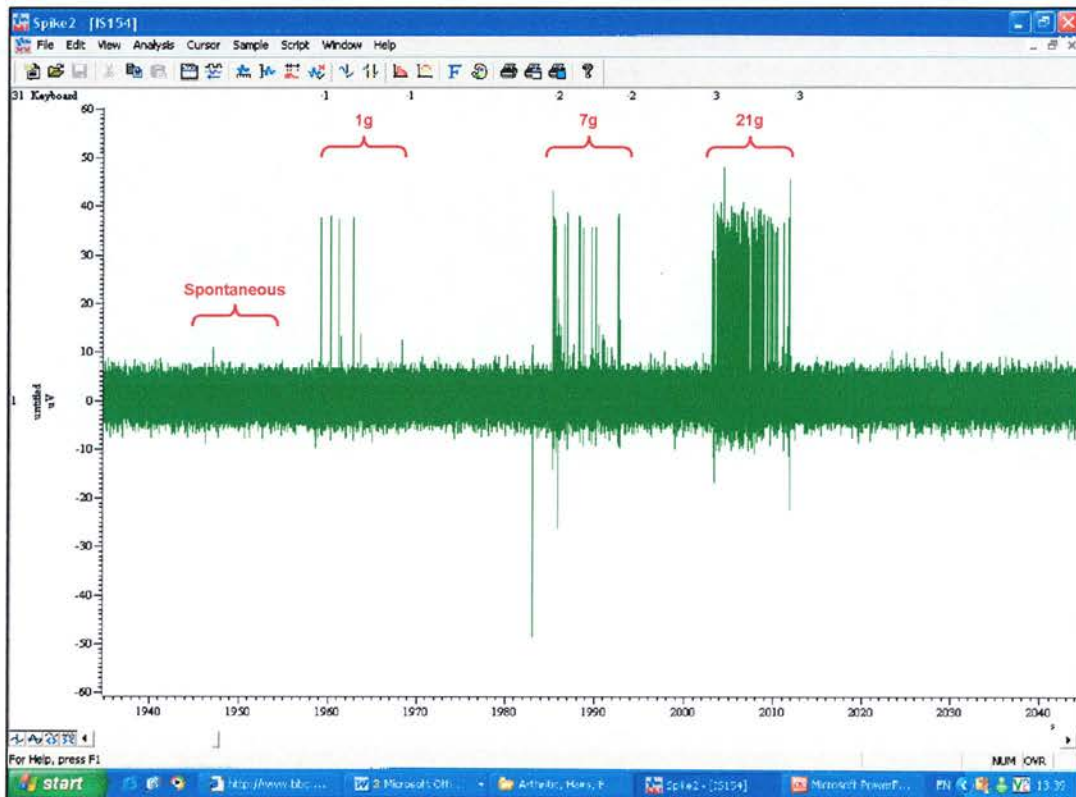


Figure 2.6 Screenshot from Spike2 of a raw filtered neuronal trace from the MAN and the responses during a typical set of mechanical stimulations using the 1g, 7g and 21g von Frey filaments. Spontaneous activity was measured for a 10s period before each individual hair was applied for 10s. The identification of individual units is shown in Figure 2.7.

2.3.6 Mechanical activity

Mechanical stimuli were applied to the surface of the knee joint exposed within the pouch using von Frey filaments (VFF, Stoelting, Scientific Marketing Associates, UK). A specific spot within the receptive field of the MAN, which was not directly over the nerve and would not disturb the nerve, was marked as the test location to ensure accurate repeat applications of the filaments. Filaments of 1, 7 and 21g were applied to the joint capsule to the point where the filament began to bend, at this time the applied force per unit area would be 14mN, 98mN and 255mN respectively. The filaments were applied by hand perpendicular to the knee in ascending order for a test period of 10s with a 10s break in between each test (see Figure 2.6). The effect of each individual filament was measured as the change in either the absolute number of action potentials or the action potential frequency (impulses.s⁻¹), between that of the test period and the preceding ten seconds.

2.3.7 Identification of nerve fibre types

Tests were performed at the end of each experiment to identify the fibre types observed throughout the experiment. In order to classify the fibres as being C-fibre a constant voltage isolated stimulator (Digitimer Ltd., UK) was used to apply an electrical stimulus within the receptive field. A series of ten single square wave pulses (1-40V at 1Hz at 0.16-1.6ms) were delivered by a stimulating electrode which was positioned at a known distance from the recording electrodes. The distance between the stimulating and recording electrodes was divided by the latency of response to calculate a conduction velocity (m.s⁻¹). As the stimulating electrode was placed so close to the recording electrodes within the boundaries of the receptive field (typically 8-12mm apart) only C-fibres could be identified as faster fibre responses were lost in the stimulus artefact. C-fibre responses were defined as those occurring 10–120ms after the electrical stimulus (conduction velocity of <1.5m.s⁻¹).

Capsaicin ($3\text{--}30\mu\text{g.ml}^{-1}$) was injected i.a. to help classify the fibre types in terms of being either positive or negative for the ion channel TRPV1, therefore allowing the fibre to be categorised as a polymodal nociceptive C-fibre.

2.3.8 The sorting of the neuronal signal into individual units using Spike 2

The raw filtered neuronal trace was saved to PC and analysed offline using the Spike2 V.5.01 software. A trigger level was set on the original trace to discriminate the spontaneous, mechanical or drug induced action potentials from the noise band (see Figure 2.7a). The experiment was then analysed from start to finish identifying any units of the same size and shape, if eight or more were identical then a waveform template for that unit was produced. Throughout the experiment several waveform templates were identified (see Figure 2.7b). The original neuronal trace was then re-scanned and the units corresponding to the different waveform templates were highlighted in different colours and separated into individual traces (see Figure 2.7c).

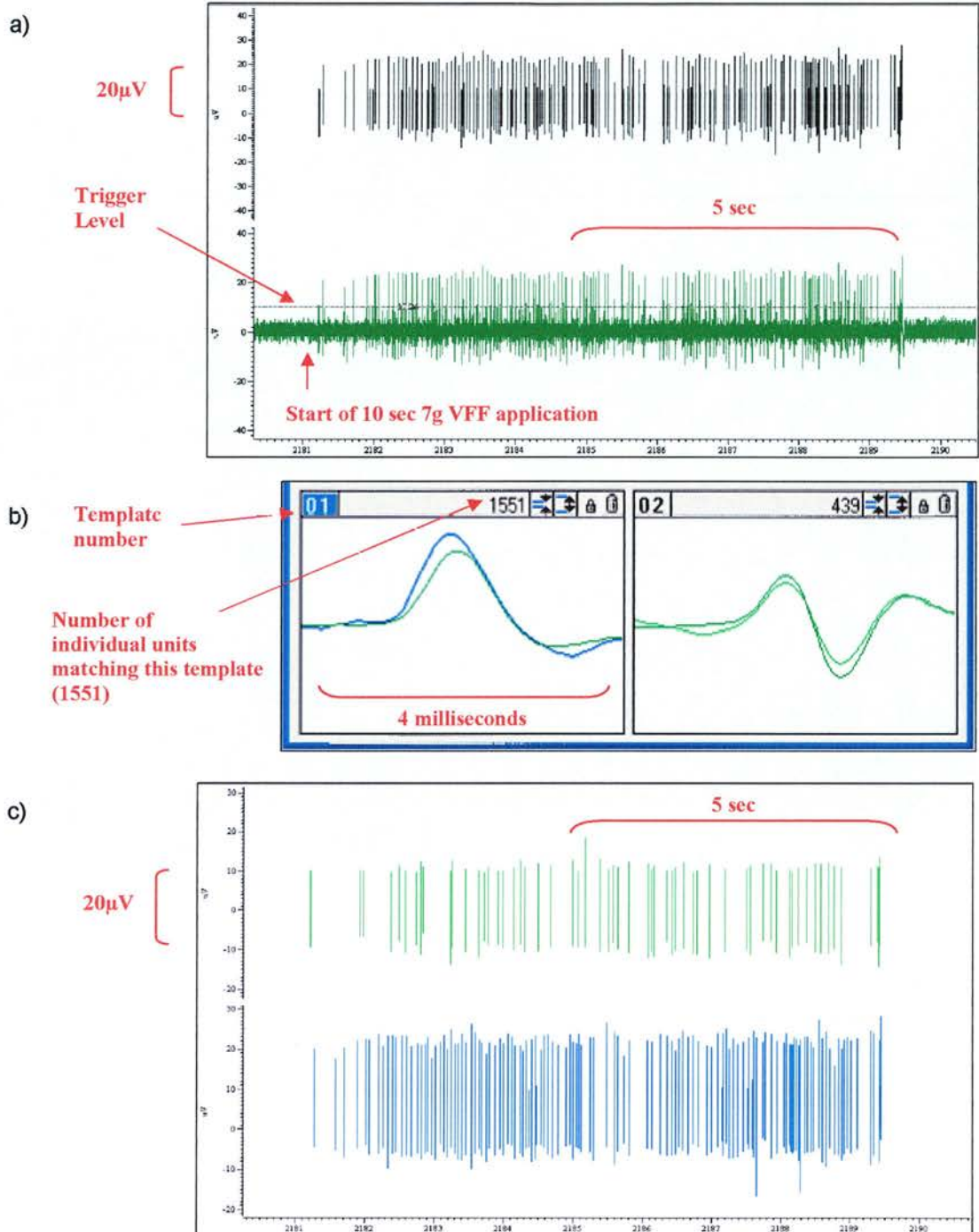


Figure 2.7 Screenshots from Spike V.5.01 software analysis on one mechanical application of the VFF for a period of approximately 10 seconds. a) The original neuronal trace (lower trace, in green) had a trigger level set to differentiate the action potentials from the background noise (top trace, in black). b) In this example two waveform templates were created from the two different units firing in response to the VFF application. Template 01 shows the waveform of a unit which fires 1551 times during this experiment (blue). The original neuronal trace was then re-scanned and the units corresponding to the different waveform templates were highlighted in different colours and separated into two individual traces c).

2.4 Immunohistochemistry (IHC)

2.4.1 Retrograde labelling of the DRG using Fast Blue

The protocol for using the dye FB as a retrograde tracer to label knee joint afferent L3-L5 DRG had been established in house at GlaxoSmithKline by Dr. Jo C. Martindale, and has also been used to study TRPV1 receptor expression (Fernihough et al. 2005). Animals were transiently anaesthetised using 3% isoflurane in oxygen, and the left (ipsilateral) knee joint was shaved and cleaned using a dilute Hibiscrub solution. Using a 30-gauge needle (BD Microfine, USA) attached to a 100 μ l Hamilton syringe; 10 μ l of 2% FB (Illing Plastics, Germany) was injected into the joint space of the left knee. Animals were allowed to recover from anaesthesia before being returned to their cage, which was layered with soft paper bedding. Trial injections were performed on cadavers prior to the study to ensure that 10 μ l of FB could be injected into the knee joint cavity without visible leakage, minimising the chance that afferent cell bodies surrounding the knee would be falsely labelled.

2.4.2 Tissue processing and sectioning

Animals were killed using a rising concentration of CO₂ followed by cervical dislocation. Ipsilateral DRGs were removed from levels L3, L4 and L5 and rinsed in phosphate buffered saline (PBS). In some cases lumbar spinal cord and brains were also removed from the animal. DRGs were immediately embedded in optimum cutting temperature (OCT, TissueTek, Canada) compound and snap frozen in isopentane cooled on dry ice. DRGs were wrapped in tin foil and carefully labelled before being stored at -80°C until they were sectioned.

Frozen DRGs were mounted on a chuck using OCT and sectioned using a Leica CM3050S cryostat. Sections were cut (12 μ m) and thaw-mounted in series on 10 slides (Superfrost Plus Gold, Menzel-Glaser, Germany) with each slide containing 5–8 sections at

different levels throughout the DRG. Slides were carefully labelled and stored in the dark at -80°C.

2.4.3 Immunohistochemistry

Sections of DRGs were prepared as previously described. Slides were removed from the freezer and allowed to air dry in the dark for one hour. Sections were then fixed in 4% paraformaldehyde (PFA) for 30 minutes. Slides were twice washed for five minutes with PBS to remove any excess PFA. The tissue sections were covered for one hour in a non-specific blocking solution of 10% normal goat serum, 10% bovine serum albumin and 80% PBS. Sections were then labelled with a specific primary antibody diluted in blocking solution and left overnight in the dark at 4°C. Additional sections were incubated with blocking solution with the omission of the specific primary antibody to act as a negative control.

Slides were removed from the refrigerator and sections were washed three times with PBS, each wash lasted five minutes. Tissue sections were covered in the appropriate secondary antibody, diluted to 1:200 in PBS, and left in the dark for two hours at room temperature. Sections were washed a further three times in PBS and covered by a coverslip using Citifluor solid mounting kit (CFPVOH and AF100, Citifluor, UK). Slides were left at room temperature to air dry, before being stored in the refrigerator prior to analysis by microscopy.

2.4.4 Antibody work-up and positive controls

Prior to commencing the TRPV1 and sodium channel IHC studies, antibody work-up was performed by investigating a range of primary antibody concentrations in order to

establish the optimum concentration of the TRPV1 and sodium channel primary antibodies to achieve the best signal to background fluorescent signal.

2.4.5 Sodium channel immunohistochemistry

The primary antibodies used were affinity isolated rabbit immunoglobulins raised against unique synthetic peptides immunogens corresponding to the sodium channels Na_v1.7 (K241), Na_v1.8 (K106), and Na_v1.9 (K186) all diluted in blocking solution 1:200. These specific alpha sodium channel subtype antibodies (GlaxoSmithKline, Harlow, UK) were all developed to cross-react with both human and rat antigens (Amaya et al. 2000; Coward et al. 2001a; b).

The secondary antibody used to reveal sites of antibody labelling was Goat anti-rabbit Alexa 488 (A11094, Molecular probes, UK) diluted 1:200 with PBS.

2.4.6 TRPV1 immunohistochemistry

Primary antibodies used were guinea-pig polyclonal TRPV1 (AB5566, Chemicon, UK) antibody diluted in blocking solution 1:1000. Levels of antibody labelling were revealed using the secondary antibody; goat anti guinea-pig Alexa 488 (A11073, Molecular Probes, UK) diluted 1:200 in PBS.

2.4.7 Microscopy and analysis

Each group contained six animals from which the ipsilateral L3-L5 DRGs were collected. Fluorescence in DRGs was observed using a Leica DMR microscope. Sections were initially observed under an excitation frequency of 380nm to reveal and count the total number of FB positive cells in 5-8 sections from each DRG. Setting the microscope filter to excite the sections at 488nm then enabled the cells labelled with the secondary antibody to

be visualised. By flicking the filters between the two different excitation wavelengths the total number of double-labelled cells were identified and counted. From the total FB count and the double labelled count, the mean animal co-localisation percentage (\pm SEM) of cells that were positive for each of the sodium channels or the TRPV1 receptors within the FB positive sub-population was calculated at each time point. Only cells with visible nuclei were counted in order to avoid duplicating the count. All analyses were carried out with the observer blind to the groupings, and representative photomicrographs were captured throughout.

2.5 **Statistical Analysis**

Data generated from behavioural, electrophysiological and immunohistochemical experiments were collated and analysed using Microsoft Excel and GraphPad Prism 4 software. Group data was expressed as the mean \pm standard error of the mean (SEM).

Statistical tests were selected according to whether the data sets being analysed were paired or unpaired, and normally distributed or not normally distributed (Motulsky 1995). Normally distributed data sets were analysed using parametric tests and non-normally distributed data were analysed using non-parametric tests. Unpaired t-tests were used to analyse the differences between the means of two normally distributed groups with similar variances. In cases where the data was not normally distributed the Mann-Whitney U-test was preferred. The Student's paired t-test (parametric) and Wilcoxon U-test (non-parametric) tests were used to compare the means of paired groups. A one-way analysis of variance (ANOVA) was used to determine whether statistically significant differences existed between three or more independent groups. If a significant difference did exist then a Dunn's multiple comparison post-hoc test was performed to determine specifically which groups were significantly different. A repeated measures ANOVA was used in cases where the groups are subjected to repeated measures such as behavioural assessments during a dosing

period. The means of three or more groups of non-parametric data were analysed with a Kruskal-Wallis test and a post-hoc analysis done using Dunn's multiple comparison.

Correlations between normally distributed groups were determined using Linear (Pearson) correlation and non-parametric data were compared using a Spearman Rank Correlation.

In all cases the null hypothesis that the observed difference between the group means or medians was due to chance was rejected at the 0.05 level. Therefore $P \leq 0.05$ was considered statistically significant and the P value was quoted to show the proximity to the 0.05 limit.

Area under the curve (AUC) analysis was used to reveal any behavioural changes in individual animals during the dosing period of the behavioural studies. A Microsoft Excel macro designed by Dr. S.T. Bate (GlaxoSmithKline), employing mathematical integration to calculate area, was used to calculate an AUC value for the change in behaviour during the dosing period for each animal. A pre-administration value of the behavioural readout (for instance WD ratio) was used as a baseline, and values recorded on subsequent days were used as recordings of how the behavioural assessment changed from day to day (Figure 2.8).

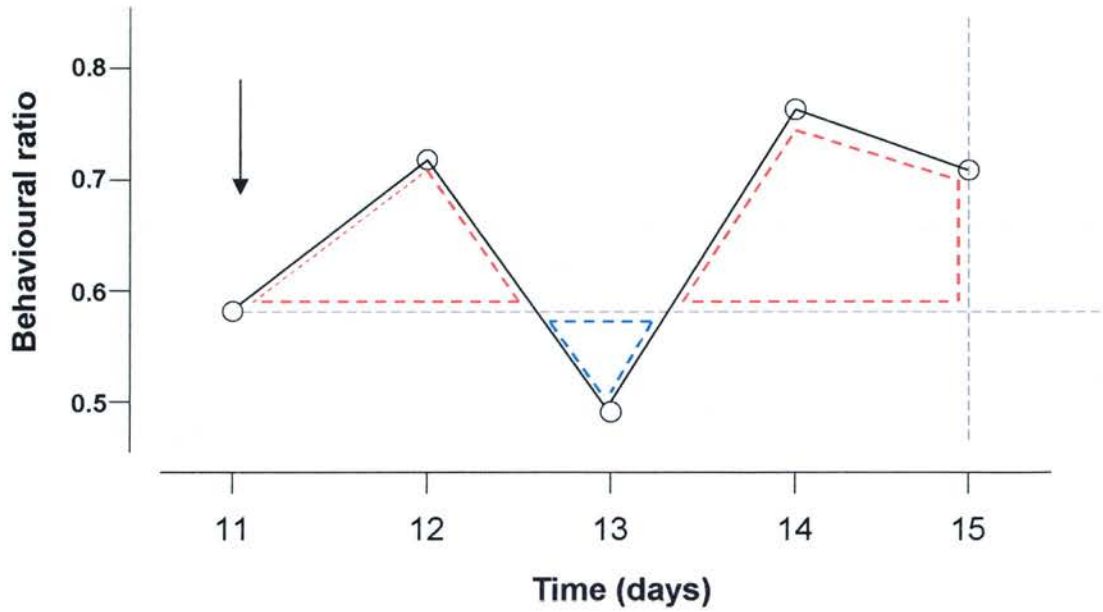


Figure 2.8 Summary diagram illustrating an example of how an AUC value was calculated using the Microsoft Excel macro. The daily assessments of the behavioural readout for an individual animal (O) were plotted during the dosing period (day 11-15). The pre-treatment value prior to dosing on day 11 was used as a baseline value and the macro calculated the areas above (red lines) as positive values, and the areas underneath (blue lines) as negative values. The individual areas were then summed to give the total AUC value for that animal in response to that drug.

Chapter 3

Neural recordings made from the Medial articular nerve (MAN)

3.1 Introduction

FCA-induced unilateral arthritis in rats has been validated as a model of human arthritis (Davis and Perkins 1993; Donaldson et al. 1993) and has been used for behavioural studies as a pre-clinical screen for novel analgesics in drug discovery (Wilson et al. 2006). Electrophysiological investigations of the primary afferent nerves in the knee provide information on the more localised mechanisms involved in the generation of nociceptive signals from the joint. Electrophysiological recordings of the MAN have historically been performed in cats (Coggeshall et al. 1983; Schaible and Schmidt 1983a; b; 1985; Mackie et al. 1995), but as it became generally unacceptable to use domestic animals in research experiments, even laboratory bred, a similar preparation has been used to study neural activity in the rat MAN (Dowd et al. 1998a; b). The rat model has been used to investigate the sensitivity of the nerve to chemical mediators and mechanosensitivity (Gauldie et al. 2001; Just and Heppelmann 2002). More recently the preparation has been used to test the antinociceptive properties of drugs administered close to the knee joint via intra-arterial injection (Hanesch et al. 2003; Gomis et al. 2004). The same electrophysiological preparation was used in this chapter and throughout this thesis to investigate discharge properties of the MAN in FCA-injected knee joints of rats with a view to understanding the mechanisms involved in the hypersensitivity observed in the conscious animal following the induction of a unilateral arthritis.

Various studies in pain research have assessed how great an applied mechanical pressure has to be in order to evoke a limb withdrawal, and how the withdrawal threshold changes with controlled changes in the experimental conditions (Hargreaves et al. 1988; Schaible and Schmidt 1988; Sluka 1997). The Randall and Selitto algometer was one of the first pieces of apparatus of its kind designed to apply a graded, measureable, mechanical stimulus to the paw of conscious animals (Randall and Selitto 1957). The paw is placed on a plinth and an increasing weight is applied to the dorsal surface of the paw. When the weight

becomes noxious the animal withdraws its paw from the apparatus and the force in grams required to evoke the withdrawal is recorded. Although the algometer is an excellent behavioural tool, the mechanics and sheer size of this apparatus do not easily enable this technique to be used on anaesthetised animals during electrophysiological recordings. Mechanical von Frey filaments (von Frey 1894a; b) are a less invasive method of testing mechanical allodynia and hyperalgesia in both behavioural (Bingham et al. 2005) and electrophysiological studies (Martindale et al. 2007). Filaments manufactured to apply a force in the range of approximately 0.5–25g have been used in the past to demonstrate the presence of tactile allodynia and hyperalgesia in the paw of animals subjected to either neuropathic or inflammatory insults (Kim and Chung 1992; Bingham et al. 2005). The use of this range of hairs for noxious and innocuous stimulation is supported by a study where it was demonstrated that the application of a mechanical stimulus greater than 15g was noxious in conscious animals eliciting a paw withdrawal from the stimulus (Chaplan et al. 1994). In animals it is very difficult to know if a withdrawal from a given stimulus is due to pain, itch or irritation. However, the threshold for withdrawal seen in rats is similar to that seen in human clinical trials in which participants report the sensation in response to this stimulus as being painful (Rolke et al. 2006a; b).

Mechanical von Frey filaments within the range of 1 to 21g (were used, in the work described in this chapter, to evoke neural discharge of the MAN nerve when applied directly onto the joint. As described in section 2.3.2 the electrophysiological technique requires the skin around the knee joint to be removed, allowing the filaments to be placed directly onto the muscle layer surrounding the knee joint.

Capsaicin is a ligand for the TRPV1 receptor and has been used throughout this thesis to trigger a nociceptive C-fibre response when given close to the knee joint via an intra-arterial injection. As discussed in section 1.2.4.2 the TRPV1 receptor is predominantly found on nociceptive C-fibres, with peripherally administered capsaicin causing acute, dose-related excitation of these fibres (Baumann et al. 1991). Healthy volunteers given an

intradermal injection of capsaicin experience an intense burning pain as the C-fibres are depolarised and a nociceptive signal is propagated (LaMotte et al. 1992). TRPV1 receptor expression has been shown to be up-regulated in knee joint afferent DRG in a rat model of OA (Fernihough et al. 2005) and in peripheral tissues following FCA-induced inflammation (Ji et al. 2002). In contrast the expression of TRPV1 remains unchanged at the 'acute' (3 days) and 'chronic' stages (21 days) of a rodent model of an antigen-induced arthritis (Bar et al. 2004). An aim of this study was to examine the expression of TRPV1 in knee joint afferent DRG cell bodies up to 28 days after intra-articular injection of FCA. This was performed as part of a larger study investigating the levels of VGSCs in naïve and FCA-injected animals and is described in much more detail in Chapter 6 of this thesis. It is appropriate to include the data set in this chapter as it relates directly to capsaicin-evoked discharge of the MAN.

The overall aim of this chapter was to compare and note the changes in the electrophysiological properties of the MAN in naïve and FCA-injected knee joints. By collating the results of all electrophysiology experiments performed, any differences in the spontaneous discharge and sensitivity of the MAN to mechanical and chemical stimulation could be detected and reported. Furthermore, using *in vivo* electrophysiology this model of unilateral arthritis was characterised prior to subsequent investigations into the role of EM1 and morphine as analgesics in experimental inflammatory joint disease.

3.2 Methods

3.2.1 Study design

Induction of unilateral arthritis was carried out 14-28 days prior to neural recordings as described in section 2.1.2. All animals were transiently anaesthetised with halothane (3% in 1.5L.min⁻¹ O₂). In total 88 animals were given an injection of FCA into the left knee joint

whereas 76 animals received no injection. On the day of the electrophysiology recordings the knee joint diameter and weight of each animal were measured. Recordings were made as described in section 2.3. Briefly rats were anaesthetised with pentobarbital sodium ($60\text{mg}\cdot\text{ml}^{-1}$ i.p.) and the trachea, right carotid artery and right femoral artery and vein were cannulated. A continuous i.v. infusion of 40% pentobarbital ($24\text{mg}\cdot\text{ml}^{-1}$ in saline) was given over the course of the experiments at approximately $325\mu\text{l}\cdot\text{hr}^{-1}$, with adjustments made based upon the individual animal's condition, to maintain a steady state of anaesthesia. The nerve was dissected from the surrounding connective tissue and placed onto the platinum/iridium wire electrodes as described in section 2.2.3.

Having placed the whole nerve bundle on the electrodes a neural recording was started. Initially the nerve was left for ten minutes in order to measure any on-going discharge. The nerve was then stimulated by $10\mu\text{g}\cdot\text{ml}^{-1}$ capsaicin (0.1ml, injected via the intra-arterial cannula), or by mechanical von Frey filaments as described in section 2.3.5 and 2.3.6 respectively. In order to help identify the fibres as either C-fibre polymodal nociceptors or type I A δ -mechanoreceptors, sensitivity to capsaicin, mechanosensitivity and conduction velocities were measured towards the end of the recording if not already performed as part of the earlier tests.

A separate study ($n=54$) was conducted to examine the expression of TRPV1 in knee joint afferent cell bodies in L3-L5 DRG of FCA-injected rats at day 7, 14, 21 and 28 post-FCA in comparison with time matched controls. Full details of the immunohistochemical methods used are described in section 2.4 with details of the antibody and concentrations used given in section 2.4.6.

3.2.2 Drugs

Capsaicin (Sigma, UK, MW= 305), was dissolved in 80% phosphate buffered solution, 10% Tween 80 and 10% ethanol (Sigma, UK). A 0.1ml injection of $3\text{-}30\mu\text{g}\cdot\text{ml}^{-1}$

solution was administered intra-arterially to evoke neural firing of TRPV1 expressing unmyelinated C-fibres.

3.2.3 Statistical analysis

To determine whether there were any statistically significant differences between the group of naïve animals and the group of FCA-injected animals, for instance when comparing body weights, a simple unpaired t-test was used to compare group mean values. In cases where statistical assessments of values obtained from the same animals were possible, such as when comparing the left and right knee joint diameters, a parametric paired t-test, or a non-parametric Mann-Whitney test, was employed as appropriate. When comparing the control and FCA-injected groups at each time point in the IHC study, a two-way analysis of variance was used as the statistical test.

3.3 Results

A total of 164 *in vivo* electrophysiology experiments were conducted during the work performed for this thesis. Of these 164 experiments, 115 (70%) were successful in obtaining usable recordings for data analysis (Figure 3.1). The 49 experiments that were not successful failed for reasons such as; the animal died during anaesthesia, the medial articular nerve was damaged in the dissection process, the blood pressure was too low resulting in inaccurate data sets and being unable to find an active nerve, despite there being no obvious technical reason such as electrode failure.

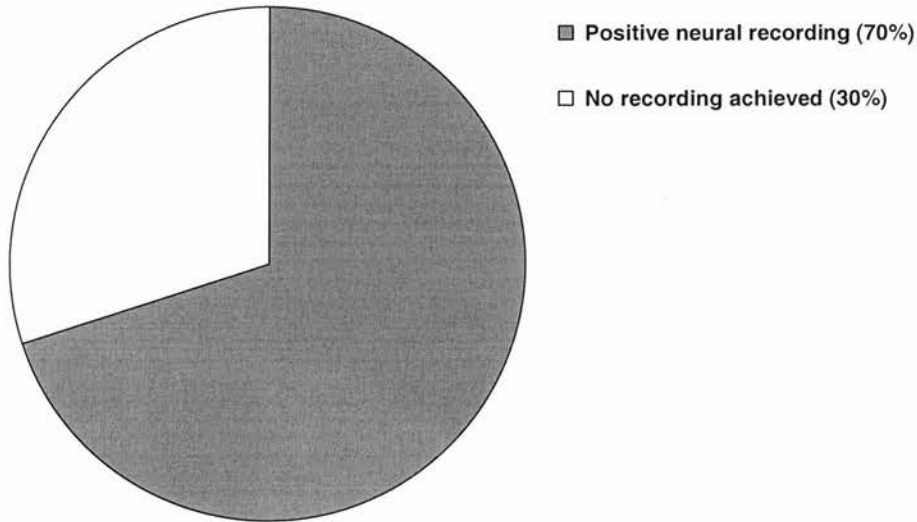


Figure 3.1 The proportion of electrophysiology experiments yielding successful recordings. Of 164 experiments started, 70% (115/164) were successful in achieving a positive neural recording from the MAN.

3.3.1 Behavioural assessments of arthritis

3.3.1.1 Body weight

The mean body weight of the FCA-injected animals was not significantly different from that of the naïve animals on the day of the electrophysiological recording ($P > 0.05$, unpaired t-test, Figure 3.2a). Animals injected with FCA continued to gain weight up until the time they were used for the recording (Figure 3.2b).

3.3.1.2 Assessment of inflammation induced by FCA

Measurement of the knee joint diameter in both naïve and FCA-injected animals revealed that the FCA-injected knee was inflamed and therefore significantly larger than the un-injected knee in the same animal, or the corresponding untreated knee in naïve animals ($P < 0.001$, one-way ANOVA, Figure 3.3a). FCA did not cause inflammation in the contralateral knee joint enabling it to be used as an internal control. The mean knee diameter

ratio of naïve animals was $1.00 \pm 0.01\text{mm}$ compared to the significantly higher value of $1.13 \pm 0.01\text{mm}$ measured for FCA-injected animals ($P < 0.001$, Mann-Whitney, Figure 3.3a). A box and whiskers plot shows there is a greater spread of knee diameter (KD) ratios in the FCA-injected animals, indicating that there is inter-animal variation in the degree of inflammation in response to the fixed FCA stimulus (Figure 3.3b).

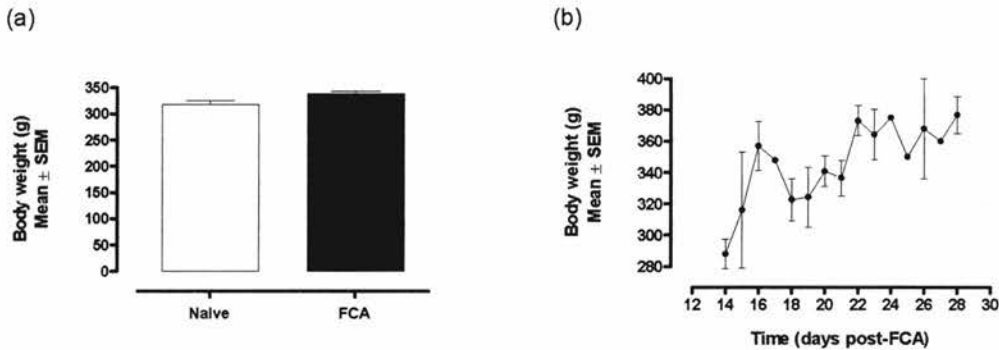


Figure 3.2 (a) The mean body weights measured on the day of the electrophysiological recording of naïve rats ($n=76$) and rats injected with FCA ($n=88$) 14-28 days prior to the recording. There was no significant difference in the mean body weights of naïve or FCA-injected animals on the day of recording ($P > 0.05$, unpaired t-test) and (b) FCA animals maintained an appropriate, gradual, increase in weight following injection of FCA indicating the animals were not experiencing any hindrance to their normal behaviours and were not in severe pain.

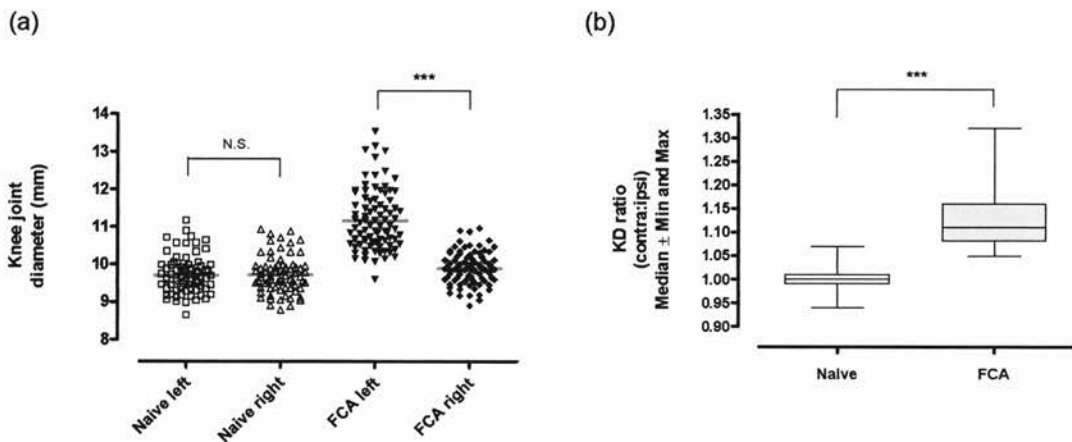


Figure 3.3 (a) Knee joint diameters of naïve ($n=76$) and FCA-injected ($n=88$) animals (red bar indicates the mean value) and (b) box and whiskers plots showing the range and median knee joint diameter ratios on the day of the electrophysiology recordings. Measurements showed that the knee joint diameter of the FCA-injected limb (left) was significantly greater than the un-injected limb ($*** P < 0.001$, paired t-test), and therefore the FCA-injected animals had a significantly higher KD ratio than naïve animals ($*** P < 0.001$, Mann-Whitney t-test).

3.3.2 Neural recordings from the MAN

Neural discharge was recorded from 118 individual afferent fibres innervating 52 naïve knee joints and 163 fibres innervating 63 FCA-injected knee joints that were spontaneously active, capsaicin positive or produced an action potential in response to stimulation of the knee joint with a rigid hand held plastic probe. In 34 out of the 52 (65%) naïve animal experiments and 50 out of the 63 (79%) FCA-injected animal experiments, capsaicin was given to help further identify the fibre types being recorded. In naïve knee joints 75% (70/93) of the fibres were mechanosensitive, capsaicin positive polymodal C-fibres, and 25% (23/93) were A δ -mechanoreceptors (Figure 3.4). This proportion did not change in FCA-injected knee joints as 97/130 (75%) of the fibres were identified as C-fibres, and 33/130 (25%) were identified as A δ -mechanoreceptors (Figure 3.4). The conduction velocity of the polymodal C-fibres was estimated as $0.97 \pm 0.09 \text{ m.s}^{-1}$ ($n=30$).

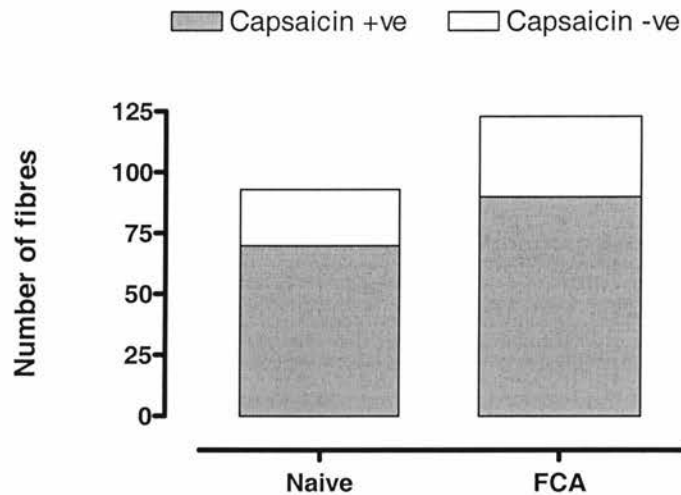


Figure 3.4 Number of fibres innervating the naïve ($n=34$, number of fibres=93) and FCA-injected ($n=50$, number of fibres=130) knee joint that were capsaicin -positive or -negative. There was no significant difference in the proportion of fibres expressing the TRPV1 receptor as 75% of both naïve and FCA-injected animals responded to capsaicin ($P>0.05$, Fisher's exact test).

3.3.2.1 Spontaneous or on-going neural discharge

In the present investigation 36% (42/118) of fibres were spontaneously active in naïve joints (Figure 3.5a) with the mean discharge frequency being 0.135 ± 0.047 impulses.s⁻¹ (Figure 3.6). In the FCA-injected joint 48% (79/163) of the fibres were spontaneously active (Figure 3.5b) with the mean discharge frequency being 0.156 ± 0.035 impulses.s⁻¹ (Figure 3.6). Both, the proportion of fibres that were spontaneously active and the mean discharge frequency of the fibres were significantly greater in FCA-injected joints than naïve ($P < 0.05$, Fishers exact test, Figures 3.5 and $P < 0.05$, t-test Figure 3.6).

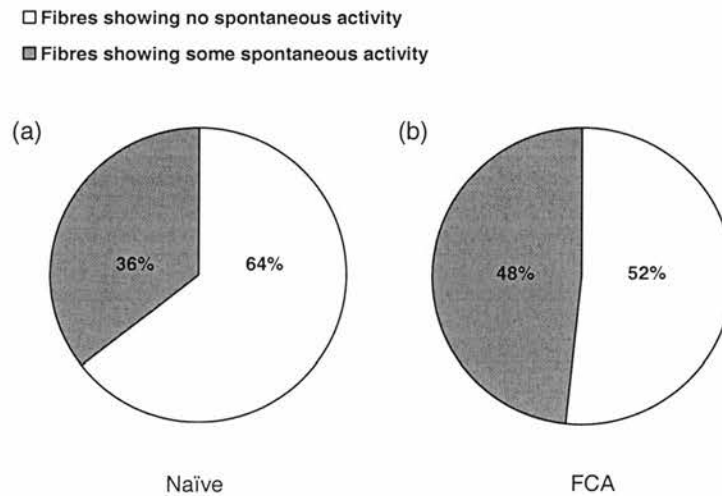


Figure 3.5 Proportions of (a) naïve (n=52, number of fibres =118) and (b) FCA-injected knee (n=63, number of fibres=163) joint afferent nerve fibres showing spontaneous neural activity in the ten minutes prior to any mechanical or chemical stimulation of the joint. There was a significantly greater proportions of spontaneously active fibres in the MAN of FCA-injected joints than naïve joints ($P < 0.05$, Fishers exact test).

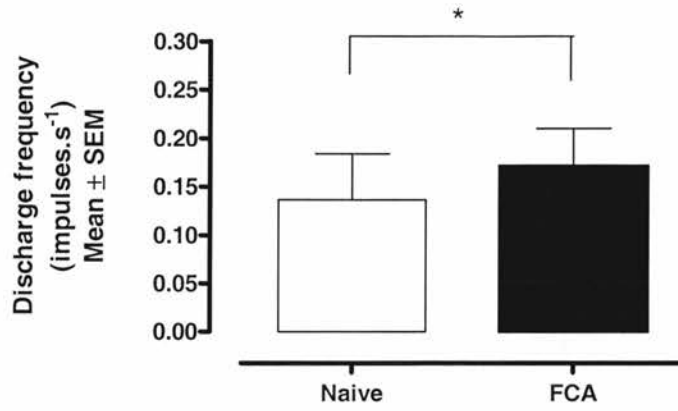


Figure 3.6 The mean spontaneous discharge frequency of fibres innervating the naïve (n=76, number of fibres=117) and FCA-injected knee (n=88, number of fibres=148) in 600s before any other tests were performed. There was significantly more spontaneous activity observed in FCA-injected knee joint afferents than there was in naïve joint afferents ($P>0.05$, unpaired t-test).

3.3.2.2 Capsaicin evoked neural discharge

In 24 naïve and 22 FCA experiments the first test to be performed following the measure of spontaneous activity was an injection of capsaicin which evoked a rapid onset, high frequency response from polymodal C-fibres innervating both the naïve and FCA-injected joints (Figure 3.7d). The mean response duration was 15.92 ± 2.67 s and 13.73 ± 2.56 s (Figure 3.7a) and the mean discharge frequency was 16.85 ± 2.41 impulses.s⁻¹ and 13.41 ± 1.96 impulses.s⁻¹ in naïve and FCA-injected joints respectively. There was no significant difference between the duration of response, the change in discharge frequency from basal frequency or change in total number of impulses from basal in naïve and FCA-injected animals ($P>0.05$, unpaired t-test, Figure 3.7a-c).

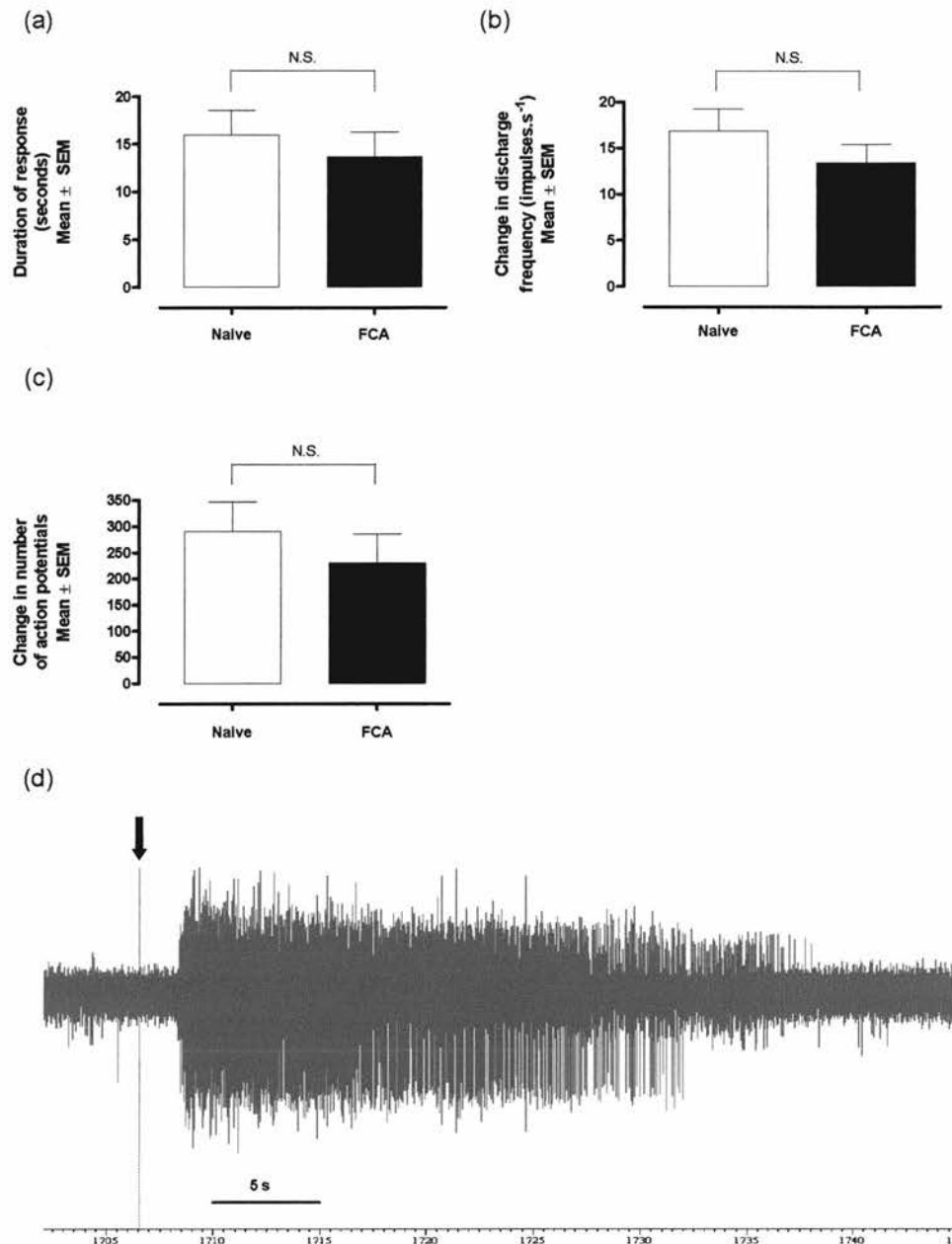


Figure 3.7 Capsaicin evoked neural discharge of fibres innervating the naïve (open rectangles) and FCA-injected (filled rectangles) knee joints. There was no significant difference between the naïve and FCA-injected animals when examining (a) the duration of response, (b) the change in discharge frequency and (c) the change in absolute number of action potentials evoked by the injection 1µg capsaicin ($P>0.05$, unpaired t-test). (d) A screenshot of a typical response of the MAN following injection of 1µg capsaicin into an FCA-injected animal at day 18 post-FCA. The arrow indicates the time at which the drug was injected.

3.3.2.3 Mechanically evoked neural discharge

In 18 naïve and 23 FCA experiments the first test to be performed following the 10min measurement of spontaneous activity was to use a range of von Frey filaments to evoke firing in the MAN. Filaments of 1, 7 and 21g caused the MAN fibres to fire when applied to the knee joint capsule (Figure 3.8a-f). There was a significantly larger response to the ten second application of both the 7 and 21g von Frey filament in FCA-injected joints compared to naïve ($P<0.05$, unpaired t-test, Figure 3.8b and d). There was no statistical difference in the change in frequency of firing evoked by the 1g hair in FCA-injected or naïve animals ($P>0.05$, unpaired t-test, Figure 3.8a).

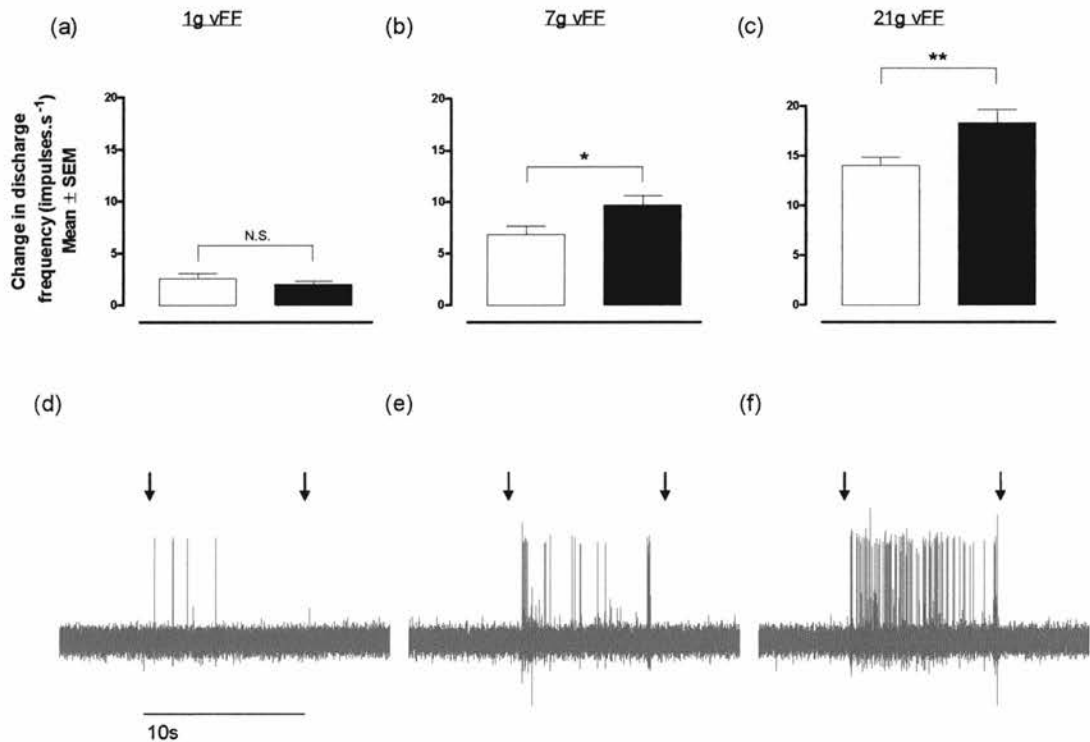


Figure 3.8 The change in frequency of neural discharge from baseline in the fibres innervating the naïve and FCA-injected knee joint evoked by (a) 1, (b) 7 and (c) 21g von Frey filaments. There is a significant increase in the response to the 7 and 21g von Frey filament in the FCA-injected joints compared to naïve ($P<0.05$, $P<0.01$, unpaired t-test). No change in frequency of firing was observed between naïve and FCA-injected animals evoked by the 1g filament. Typical responses of the nerve to stimulation by the von Frey filaments taken are shown in d-f, recordings taken from an FCA-injected animal (18 days post-FCA).

3.3.3 Immunohistochemistry

3.3.3.1 TRPV1 receptor expression

Expression of the capsaicin sensitive TRPV1 receptor was examined in a subpopulation of L3-L5 DRG cell bodies innervating the knee joint. FB was used to highlight the cell bodies of fibres innervating the knee joints and a specific TRPV1 receptor primary antibody was used to identify TRPV1 positive cell bodies. The level of TRPV1 receptor-expressing cells within the FB-positive population did not change from pre-FCA measurements at any time point studied following an intra-articular injection of FCA (Figure 3.9). Moreover there was no significant difference in the mean percentage levels of TRPV1 expression in control animals at any time point investigated when compared to FCA ($P>0.05$, two-way ANOVA). The total number of FB-positive cells analysed in this study was 798 (Group mean = 89 ± 7), with the Pre-FCA value of 38% calculated from 47 of the 125 FB-positive cells expressing the TRPV1 receptor.

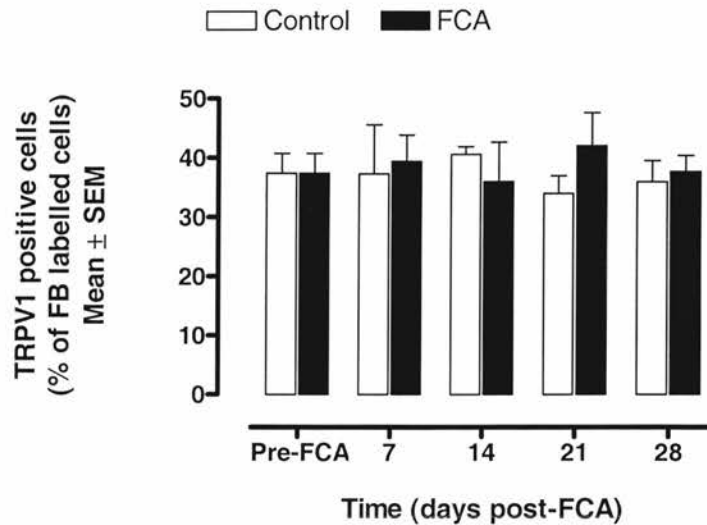


Figure 3.9 Quantitative analysis showing the percentage of FB cells in ipsilateral L3-L5 DRG which expressed the TRPV1 receptor, measured at various time points following injection of 2% FB (10 μ l, control) or FB + FCA (150 μ l, 1mg.ml⁻¹, FCA) into the left knee joint. Control animals are defined as those receiving only FB. There was no significant difference in the percentage of FB cells expressing TRPV1 in control or FCA-injected animals at any time point ($P>0.05$, two-way ANOVA).

3.4 Discussion

Electrophysiological recordings showed the spontaneous, mechanical and chemical properties of the MAN in naïve and FCA-injected animals. Out of 164 experiments conducted, over 70% were successful in obtaining a positive neural recording. Guidelines on the use of pentobarbital sodium in small animals were followed to prevent unwanted adverse effects, but unfortunately on some occasions even a small overdose led to complications such as a fatal fall in blood pressure or a rapid loss in body temperature, making the animal unviable and the data collected unusable. Occasionally a nerve would be dissected and placed on the electrodes and it appeared that there were no active fibres in the nerve bundle. In preparations where this occurred the electrodes were replaced and the recording equipment was checked for faults. Following these alterations, if there were still no active units seen then a further branch of the nerve would be dissected and investigated. Dissection of the nerve was a more difficult process in the inflamed joints compared to the naïve joints. FCA caused an increase in blood vessels around the joint, increasing the risk of bleeding, with the local oedema making the joint capsule and surrounding area a less rigid structure. Damage to the MAN during the dissection process and anaesthetic attributed effects were the main reasons for an experiment being unable to produce a positive recording. With respect to the previously mentioned complications, a success rate of approximately 70% in both naïve and FCA-injected animals can be considered a commendable achievement in this experimental protocol, comparing favourably with previous experimenters in the laboratory (McQueen, oral communication).

To ensure the fibres investigated were involved in nociceptive signalling, a noxious mechanical stimulation was applied to the knee joint capsule using a plastic probe and identification of the fibre types using capsaicin and conduction velocity tests were later performed. Studies from the cat knee joint suggest that only 70% of C-fibres and 55% of A δ -fibres are nociceptive and therefore clear identification in these studies is essential to ensure

the fibres investigated were nociceptive (Schaible and Schmidt 1983b). Unfortunately conduction velocities of A δ -fibres were unobtainable as the stimulating electrodes were in such close proximity to the recording electrodes that the A δ -responses were lost in the stimulus artefact. Therefore it was presumed that the fibres responding to noxious mechanical stimulation, but not providing a measureable conduction velocity were A δ -mechanonociceptors. Identification of the neural profile of the MAN revealed that C-fibres accounted for 75% of the fibres studied with A δ -fibres making up the other 25%, a value that was the same in both naïve and FCA-injected joints. These results are in accord with values obtained from previous studies conducted in this laboratory where 61% (Dowd et al. 1998b) and 93% (Gauldie et al. 2001) of the fibres were identified as C-fibres, and histological studies in the cat knee joint where 80% of the afferents were identified as unmyelinated C-fibres (Langford and Schmidt 1983). No efferent neural activity was recorded during these experiments as the sciatic nerve was cut proximally from the recording to prevent any descending signals.

Results from the present study show that there were a greater proportion of spontaneously active MAN fibres, discharging at a higher frequency in FCA-injected knee joints than in naïve knee joints. This is a common characteristic observed in nociceptors innervating inflamed ankle and knee joints of varying species (Guilbaud et al. 1985; Schaible and Schmidt 1985; McQueen et al. 1991). Another feature of the FCA-injected knee joint fibres in this study is that they show an increased responsiveness to high threshold mechanical stimulation using von Frey filaments when compared to naïve fibres. In behavioural studies von Frey filaments of 15g and above placed on the paw of rats are described as noxious as they cause repeated limb withdrawals (Chaplan et al. 1994). This is a finding that correlates with clinical studies where patients describe the same 15g stimulus as being painful (Rolke et al. 2006a; b). In the present study filaments of 1, 7, and 21g (14, 98 and 255mN respectively) were placed directly onto the muscle surrounding the knee, with any protection that the skin supplies as a barrier being absent. In this respect it may not be

unreasonable to consider the 7g hair as being a potentially noxious stimulus. The only way to confirm this would be to perform a behavioural study in which von Frey filaments would be applied on the knee joint of conscious animals with the skin surrounding the knee joint removed, in order to determine the behavioural withdrawal threshold. This is clearly not a feasible study to perform as the animals would be susceptible to a high risk of infection and moreover the procedure would not be allowed by Home Office regulations. Therefore the true nature of the 7g stimulus can only be speculated upon. Increased responsiveness to mechanical stimuli has been previously reported in a cat model of joint inflammation, occurring as a result of an increase in afferent discharge, and a spinal sensitisation resulting from this persistent afferent input (Neugebauer and Schaible 1990).

The response of the MAN fibres in naïve and FCA-injected joints to the administration of capsaicin did not differ in terms of the duration; change in frequency discharge or in the total number of impulses evoked. These results are consistent with previous PhD studies in this laboratory, with the values being approximately the same and with no changes being observed in the properties of capsaicin evoked neural firing in naïve or FCA-injected animals (Dowd 1999). Inflammatory pain studies in mice, rats and guinea-pigs report that capsazepine was ineffective in attenuating mechanical hyperalgesia produced by an FCA-induced paw inflammation (Walker et al. 2003). Previous IHC studies have revealed that the TRPV1 receptor can be up-regulated in knee joint afferent DRG in a model of osteoarthritis (Fernihough et al. 2005). Conversely a recent study described no change in TRPV1 receptor expression at an early or late stage during a model of antigen-induced arthritis (Bar et al. 2004). Studies conducted that are reported in the present study concur with the later study, as there was no apparent change in the expression of TRPV1 in a distinct population of DRG cell bodies of afferents innervating the knee joint, up to 28 days post-FCA. The fact that the TRPV1 receptor levels remain the same in naïve and FCA-injected animals is consistent with the electrophysiological results obtained, which showed

no alteration in response to capsaicin in the FCA-injected joints in comparison with naïve joints.

The intra-articular injection of FCA is a well studied pre-clinical model of human arthritis and chronic pain (see section 1.4.10). The injection of FCA had no adverse effects, other than the expected joint swelling, on the animals used in these electrophysiological investigations. This was evidenced by the fact that FCA-injected animals continued to gain weight consistently, and showed no significant difference in weight compared to naïve animals on the day of recording. This further confirms that the model only induces a mild arthritis in the injected joint, without the systemic effects described in less refined FCA models (Casey and Dubner 1989). In addition FCA caused a significant swelling in the injected knee compared to the un-injected knee in the same animals, or the knees of naïve animals.

The main aim of these studies was to compare the electrophysiological properties of the MAN innervating the FCA-injected knee joints with those of the naïve knee joints. Results are consistent with previous studies performed in this laboratory and therefore no further validation of the FCA-model and the recording technique is required, allowing a reduction in the number of animals used in this thesis. The data indicates that the preparation provides a viable method for recording nociceptive C- and A δ -fibres innervating the knee joint. Furthermore this electrophysiological preparation can be confidently used in order to study the antinociceptive properties of EM1 and morphine which are discussed in a later chapter. Using capsaicin evoked nociceptive neural activity and the quantitative mechanical von Frey filament stimulation, any change in discharge patterns in the MAN following EM1 and morphine administration should show any peripherally driven antinociceptive properties of the drugs.

In conclusion, this pilot study verified that FCA induces a mild inflammatory knee joint hypersensitivity in rats which occurs in part as the result of an increase in spontaneous neural discharge and a hypersensitivity to mechanical stimulation of the MAN.

Chapter 4

The role of peripheral opioid receptors in joint pain

4.1 Introduction

The primary palliative aim when treating RA is to provide adequate management of the chronic pain associated with the disease and therefore improve the patient's quality of life (Heiberg and Kvien 2002; Heiberg et al. 2005). The long term use of COX-2 inhibitors has been questioned following the discovery that the use of these compounds may lead to an increased chance of the patient suffering cardiovascular complications such as a myocardial infarction or thrombotic stroke, due to the drugs preventing formation of PGI₂ which has been shown to inhibit platelet aggregation, cause vasodilation and prevent smooth muscle proliferation in the vasculature (Fitzgerald 2004). Opioids often provide excellent analgesia to a high number of patients; however just like the COX-2 inhibitors, the long term use of opioids is hampered by the intolerable side-effects resulting from receptors located in the central nervous system and viscera (section 1.5.4.1). Analgesia, together with the associated side-effects, has been observed in animal models of FCA-induced arthritis, highlighting a positive translation of actual clinical symptoms into a successful pre-clinical model (Walker et al. 1996). Restricting the administration of these drugs to the site of injury in the periphery, for instance in or around the knee joint in the case of RA, may provide similar analgesia to that observed with systemic administration of the drugs, with minimal undesirable side-effects. Knee joint pain, as assessed using the visual analogue scale (VAS) and pain rating indices (PRI), was reduced in patients treated with low doses of intra-articular morphine following knee joint arthroscopy (Stein et al. 1991; Joshi et al. 1993). Furthermore, intra-articular morphine provided excellent analgesia to RA and OA patients, without any adverse side effects (Likar et al. 1997; Stein et al. 1999), and there is compelling evidence that the analgesia is mediated through opioid receptors located on primary afferent neurons (see section 1.5.5.1).

The endogenous opioids EM1 and EM2 are present in human immune cells known to migrate to sites of tissue injury. The expression of EM1 and EM2 has been shown to be

increased in the synovial tissue taken from inflamed hind paws and the spleen and thymus when compared to control animals in a rat model of adjuvant-induced arthritis (Jessop et al. 2002). This suggests that the endogenous opioids play an important role in an in-built mechanism for providing analgesia at sites of tissue inflammation. Exogenous EM1 given into the knee joint has been shown to reduce synovial protein extravasation and increase knee joint vascular resistance (Barin and McDougall 2003; McDougall et al. 2004a). Both of these effects are mediated through opioid receptors present on capsaicin-sensitive unmyelinated fibres, suggesting the action is via a neurogenic mechanism which inhibits the release of the pro-inflammatory mediators SP and CGRP. The same group (McDougall et al.) report that a build up of EM1, which occurs in the knee joint during the initial inflammation, results in a down-regulation of the μ -opioid receptor and a subsequent loss of the effects of exogenously administered EM1. In these studies IHC revealed a reduction in the expression of both EM1 and the μ -opioid receptor in the rat knee synovium and the primary afferent fibres innervating the inflamed knees 1 week after FCA-injection (McDougall et al. 2004b). Furthermore, the antinociceptive effect of EM-1 that is observed in normal knee joints following hyper-rotation of the knee was absent in animals at 1 week post-FCA (Li et al. 2005). These findings provide compelling evidence that exogenous μ -opioid receptor agonists, such as EM1 and morphine, should not cause analgesia when administered directly into the knee joint. However as seen from the clinical evidence described above, low doses of morphine do in fact provide excellent analgesia in patients suffering from inflammatory arthritis. The difference between the pre-clinical model and the actual clinic scenario could cast doubts over the validity of the rat model as a correlate of human RA. However, it may simply be the case that in the rat model of monoarthritis, at one week post-FCA the initial inflammatory drive is still very strong and has not yet subsided, accounting for the observed loss in opioid receptor mediated antinociception. It may actually be more appropriate to study times later than one week post-FCA when aligning this pre-clinical model with the clinical condition.

4.1.1 Hypothesis

The aim of this study was to test the hypothesis that functional opioid receptors are expressed on rat knee joint nociceptors within the MAN at times later than one week post-FCA. To do this, behavioural studies were performed to investigate the effect of intra-articular EM1 and morphine, on the way the animals distribute their weight over the FCA-injected and non-injected hind limbs. In two separate studies, three doses of EM-1 were administered on days 14, 16 and 18 post-FCA, and a single dose of morphine was administered on day 14 post-FCA, directly into the FCA-injected knee joint under brief halothane anaesthesia and any changes in the hind limb weight distribution were recorded.

Electrophysiology studies were also separately performed to investigate the effect of EM1 and morphine on the neural discharge in nociceptors recorded from the MAN evoked by mechanical von Frey filaments and capsaicin. Mechanical hyperalgesia and allodynia of the joint is often observed in the clinic, with patients reporting tenderness of the joints to touch and movement. Dosing with capsaicin is a clinically relevant way of investigating if EM1 and morphine alter the neural discharge evoked by capsaicin, as capsaicin creams such as Axsain are used in the clinic as topical analgesics (Rains and Bryson 1995) and the mechanism of action of the agonists is mediated through TRPV1 receptors present on unmyelinated C-fibres.

4.2 Methods

4.2.1 Design of behavioural studies

Throughout the behavioural experiments the body weights of the animals were recorded and their general behaviour observed to ensure that the animals were not suffering any adverse effects following administration of the test drugs or FCA.

4.2.1.1 Behavioural assessment of intra-articular morphine

On the first day of the study (day 0) the weight distribution ratio of 32 adult male Wistar rats was assessed using the rat incapacitance tester, as described in section 2.2.1. Following this assessment all the animals were anaesthetised (3% halothane in 1.5l.min⁻¹ O₂) and received an intra-articular injection of 150µl of 1mg.ml⁻¹ FCA into the left knee joint as described in section 2.1.2. On days 4, 7, 11 and 14 post-FCA, weight bearing assessments were made to confirm that the expected FCA-induced unilateral joint hypersensitivity had been induced. The animals were ranked and randomised into four dosing groups (n=8) based on the day 14 weight distribution ratio. The animals were then briefly anaesthetised (3% halothane in 1.5l.min⁻¹ O₂) and a second injection of either morphine sulphate (1, 3 or 10mg in 0.1ml) or vehicle (saline) was administered into the left knee joint. All drugs were dosed blind and the code was not revealed until the entire data set had been processed. Animals were allowed to recover in their cages before further behavioural measures of the weight distribution were made at 30, 60, 90, 120 and 180 minutes post drug injection. Further weight distribution assessments were made on days 18 and 21 post-FCA.

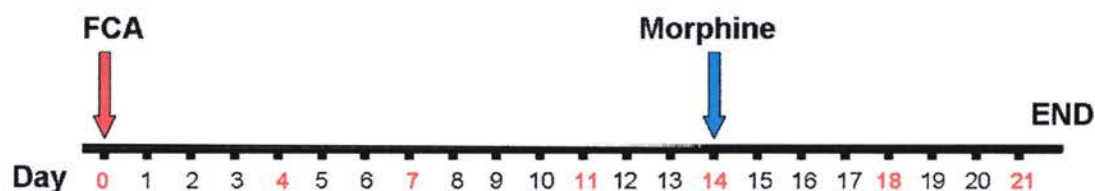


Figure 4.1 Figure illustrating the protocol for the morphine behavioural study. Intra-articular injection of FCA or morphine (1, 3 or 10mg.ml⁻¹) was administered as indicated by the red and blue arrows respectively. The days highlighted in red are the days on which behavioural measurements were made.

4.2.1.2 Behavioural assessment of intra-articular EM1

On the first day of the study (day 0) the weight distribution ratio of 32 adult male Wistar rats was assessed using the rat incapacitance tester, as described in section 2.2.1. All animals then received 150µg of FCA into the left knee joint as described in section 2.1.2. On

days 1, 3, 7, 9 and 11 post-FCA, weight bearing assessments were made to confirm the expected FCA-induced unilateral joint hypersensitivity. The animals were then ranked and randomised into four dosing groups ($n=8$) based on the day 11 weight distribution ratio. On days 14, 16 and 18 the animals were briefly anaesthetised (3% halothane in $1.5\text{ l}\cdot\text{min}^{-1}\text{ O}_2$) and on each occasion received a further injection of $100\mu\text{ l}$ of EM1 (30, 100 or 300 mg in 0.1 ml) or vehicle (saline) into the left knee joint. On each of these three occasions the animals recovered in their cages before a further weight distribution assessment was performed 30 minutes post-drug injection. Further weight distribution assessments were made on days 21 and 23 post-FCA.

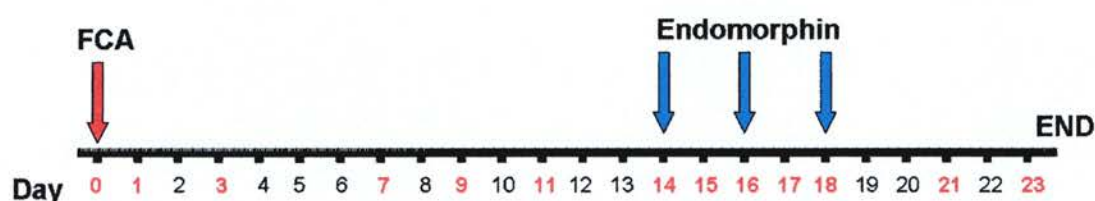


Figure 4.2 A schematic diagram illustrating the protocol for the endomorphin behavioural study. Intra-articular injection of FCA (red arrow) or endomorphin (blue arrow, 1, 3 or $10\text{ mg}\cdot\text{ml}^{-1}$) was administered on the days indicated by the arrows. The days highlighted in red are the days on which behavioural measurements were made.

4.2.1.3 Design of experiments involving neural recordings

Induction of a unilateral arthritis was carried out 14–28 days prior to neural recordings as described in section 2.1.2. Animals were briefly anaesthetised and received either an injection of $150\mu\text{ l}$ of FCA ($1\text{ mg}\cdot\text{ml}^{-1}$) into the left knee joint or were untreated. Animals receiving no injection are defined as ‘naïve’ throughout this chapter. Animals were prepared for a neural recording under terminal anaesthesia as described in section 2.3. Briefly rats were anaesthetised with pentobarbital sodium ($60\text{ mg}\cdot\text{ml}^{-1}$ i.p.) and the trachea, right carotid artery and right femoral artery and vein were cannulated. A continuous i.v. infusion of 40% pentobarbital ($24\text{ mg}\cdot\text{ml}^{-1}$ in saline) was given over the course of the experiments at approximately $325\mu\text{ l}\cdot\text{hr}^{-1}$ to maintain a steady state of anaesthesia, which was

adjusted according to the needs of the individual animal to ensure a consistent level of anaesthesia throughout. Arterial blood pressure was recorded throughout the experiment, and any changes in blood pressure following administration of a drug were calculated. The MAN was dissected from the surrounding connective tissue and placed onto the platinum/iridium wire electrodes as described in section 2.2.3. Having successfully positioned the nerve across the electrodes a neural recording was started. All drugs were administered intra-arterially in a volume of 0.1ml, as described in section 2.3.5. At the end of each experiment conduction velocities were obtained from the recorded units to help further classify the fibres.

4.2.1.4 Capsaicin evoked neural discharge

Control experiments were performed in both FCA-injected (n=6) and naïve (n=5) animals to ensure that repeat doses of capsaicin could be given without desensitising the MAN. Five doses of capsaicin (1µg) were given at 45 minute intervals. The change in discharge frequency of the MAN fibres (impulses.s⁻¹) from the background caused by the injected capsaicin was recorded. Between each dose of capsaicin two saline washes were given to thoroughly clear the cannula and remove any excess drug from the system. A saline injection was given as a vehicle control injection for the EM1 and morphine.

4.2.2 Effect of morphine and EM1 on neural discharge evoked by capsaicin

The effect of morphine and EM1 on the capsaicin evoked firing of the MAN was investigated in both FCA-injected (morphine n=6, EM1 n=5) and naïve (morphine n=5, EM1 n=6) animals. The protocol followed is shown in Figure 4.3. Briefly the first two doses of 1µg capsaicin were given with a 45 minute interval, with a further 45 minute break at which point a single dose of 0.3mg of morphine or 0.1mg of EM1 was injected. The next dose of capsaicin was given 20mins after the drug, with the final doses again being 45 minutes apart.

The change in discharge frequency of the MAN fibres (impulses.s⁻¹) from the background activity was measured following the administration of all the drugs.

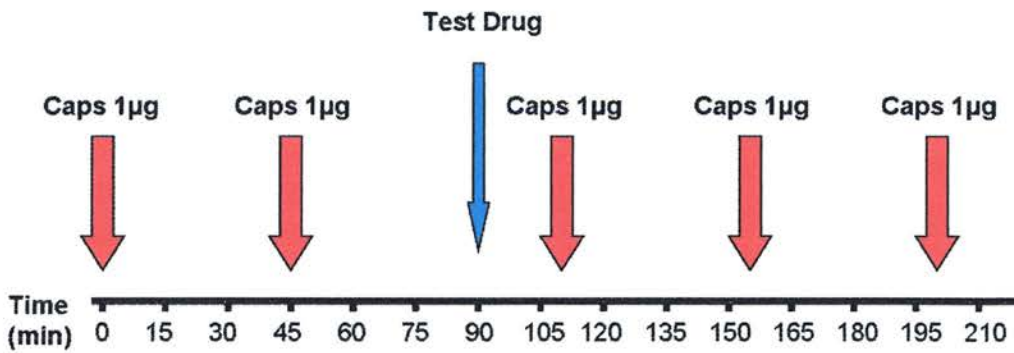


Figure 4.3 Schematic of the test protocol used in the capsaicin-evoked neural discharge experiments. The times that the close arterial injections of capsaicin (red arrows) and EM1 or morphine (blue arrow) were given are shown.

4.2.2.1 Mechanically evoked neural discharge

Control experiments were performed in both FCA-injected (n=5) and naïve (n=6) animals to ensure that repeated stimulation of the MAN by mechanical von Frey filaments produced consistent responses. For each test the von Frey filaments of 1, 7 and 21g were applied in ascending order as described in section 2.3.6. Ten tests were performed at 20 minute intervals with the administration of a vehicle control injection (saline) 15 minutes after test 5 (see Figure 4.4). The change in discharge frequency of the MAN fibres (impulses.s⁻¹) from the background activity was measured following the application of the von Frey filaments and administration of saline.

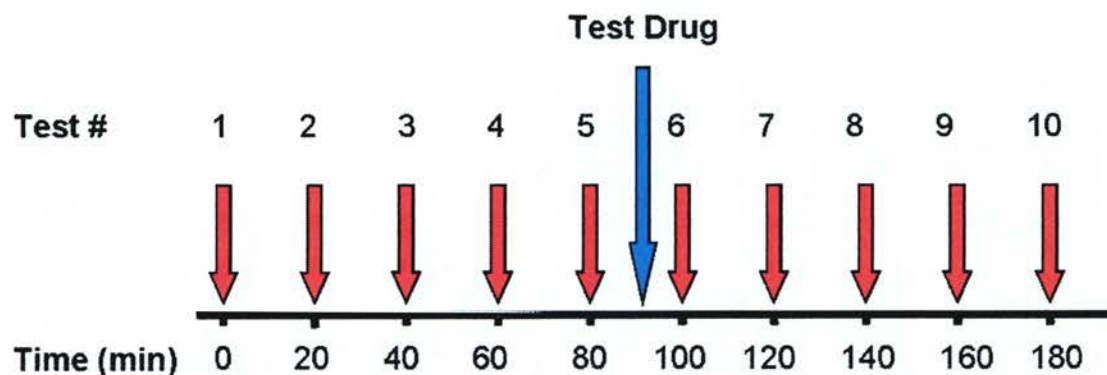


Figure 4.4 Schematic showing the test protocol showing the times of mechanical stimulation (red arrows) and the time when either EM1 or morphine were delivered (blue arrow). Pre-treatment with naloxone (500 μ g) in certain experiments occurred 3 minutes before the test drug was delivered.

4.2.2.2 Effect of morphine and EM1 on mechanical evoked neural discharge

The effect of morphine and EM1 on the mechanically evoked firing of the MAN was investigated in both FCA-injected (morphine $n=5$, EM1 $n=5$) and naïve (morphine $n=6$, EM1 $n=6$) animals. Five mechanical tests were performed before and after dosing of administering morphine (300 μ g) or EM1 (100 μ g, Figure 4.4). The change in discharge frequency of the MAN fibres (impulses. s^{-1}) from the background activity in response to each von Frey filament was measured before and after the administration of all the drugs.

To investigate whether the effect of morphine and EM1 was mediated through an opioid receptor mechanism further experiments were performed in FCA-injected animals using naloxone (morphine $n=4$, EM1 $n=4$). Naloxone (500 μ g), the opioid receptor antagonist was administered 3 minutes before either morphine or EM1 to antagonise the action of the drug at the opioid receptors.

4.2.3 Drugs

Morphine and EM1 were administered directly into the knee joint in a volume of 100 μ l during the behavioural experiments. It was assumed that 100 μ l of the injected drug remained within the knee joint cavity and was immediately diluted 1:1 by the 100 μ l of

synovial fluid already present in the FCA-injected knee (Barton 2007). Morphine sulphate salt pentahydrate (Sigma, UK, MW=758.8) was dissolved in sterile saline and administered at concentrations of 1mg.ml^{-1} , 3mg.ml^{-1} and 10mg.ml^{-1} . These doses resulted in a maximum dose of 0.1, 0.3 and 1mg of compound being administered into the knee respectively. These doses were chosen as they were comparable with those used in behavioural studies in the same model where rats weighing approximately 300g received a 3mg.kg^{-1} oral dose twice daily, a protocol which gave a significant analgesic effect (Wilson et al. 2006). The more potent μ -opioid receptor agonist EM1 (Sigma, UK, MW=610.7) was dissolved in sterile saline and administered at concentrations of $30\mu\text{g.ml}^{-1}$, $100\mu\text{g.ml}^{-1}$, $300\mu\text{g.ml}^{-1}$, with the maximum amount of drug present in the knee being 3 μg , 10 μg and 30 μg respectively.

In the *in vivo* electrophysiology studies, capsaicin (Sigma, UK, MW= 305) was dissolved in 80% phosphate buffered solution, 10% Tween 80 and 10% ethanol (Sigma, UK). A dose of 1 μg was chosen based upon previous studies in the laboratory where 1 μg of capsaicin delivered intra-arterially evoked neural discharge without desensitising the fibres. Morphine and EM1 were prepared as described above and injected at a concentration of 300 μg and 1 μg respectively following the results obtained from the behavioural studies.

4.2.4 Statistical analysis

- Individual group mean values were analysed for statistical differences using a Wilcoxon t-test or an un-paired t-test depending on whether the data were paired or un-paired respectively.
- Results obtained from the behavioural studies were expressed as the group mean (\pm SEM) for the WD ratios.
- The statistical test used to compare differences within the same group over time was a one-way ANOVA followed by Dunn's post-hoc test, as appropriate.

- The test used to compare differences between two or more different groups over time was the two-way ANOVA followed by Bonferroni's post-hoc test.
- In order to compare the AUC values calculated from each dose of drug over the dosing period, a Kruskal-Wallis ANOVA was used followed by Dunn's post-hoc to compare the different doses with the vehicle.
- The statistical test used to compare the group mean neural discharge frequencies evoked at different time points, or by the different von Frey filaments in the electrophysiology studies was a one-way ANOVA followed by Dunn's post-hoc test.
- In all cases a P value of ≤ 0.05 was considered statistically significant.

4.3 **Results**

4.3.1 **Behaviour**

The injection of FCA into the left knee joint did not have any detrimental effect on the animals' general behaviour over the duration of the morphine and EM1 studies. Despite a small decrease in weight on day 1 following the injection of FCA, animals gained weight on everyday post-FCA, a clear indication that the animals were not in severe pain and were able to perform normal behaviours such as feeding, grooming and drinking. Moreover, neither the single injection of morphine (Figure 4.5) nor the three injections of EM1 (Figure 4.6) into the knee joint prevented the animals from continuing to gain weight. Furthermore there was no significant difference in the group mean body weights for the different groups dosed with morphine (0.1-1mg) or EM1 (3-30 μ g, $P > 0.05$, two-way ANOVA).

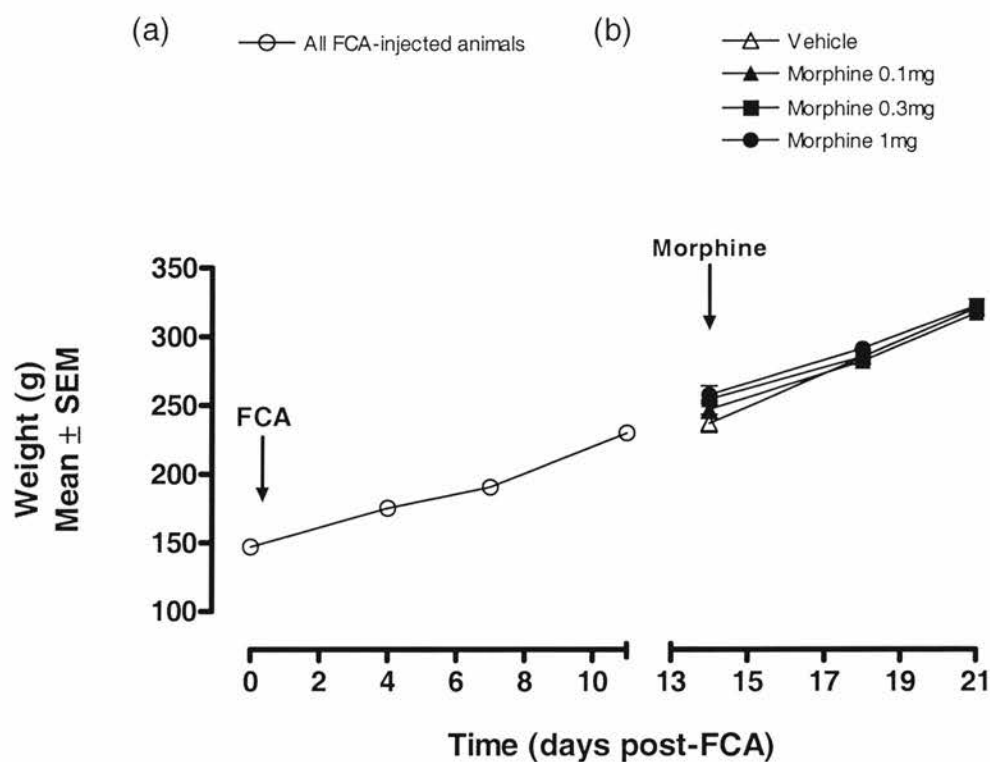


Figure 4.5 Figure illustrating (a) the pooled mean body weights for all rats (n=32, days 0-11) and (b) the same set of animals shown in their designated dosing groups for morphine (n=8, days 14-21) following injection of FCA on day 0. Animals gained weight daily over the duration of the study period. Intra-articular injection of morphine (0.1, 0.3 and 1mg) on day 14 had no effect on the weight gain of the animals and consequently there was no significant difference in the group mean body weights on days 14 to 21 ($P>0.05$, two-way ANOVA, n=32).

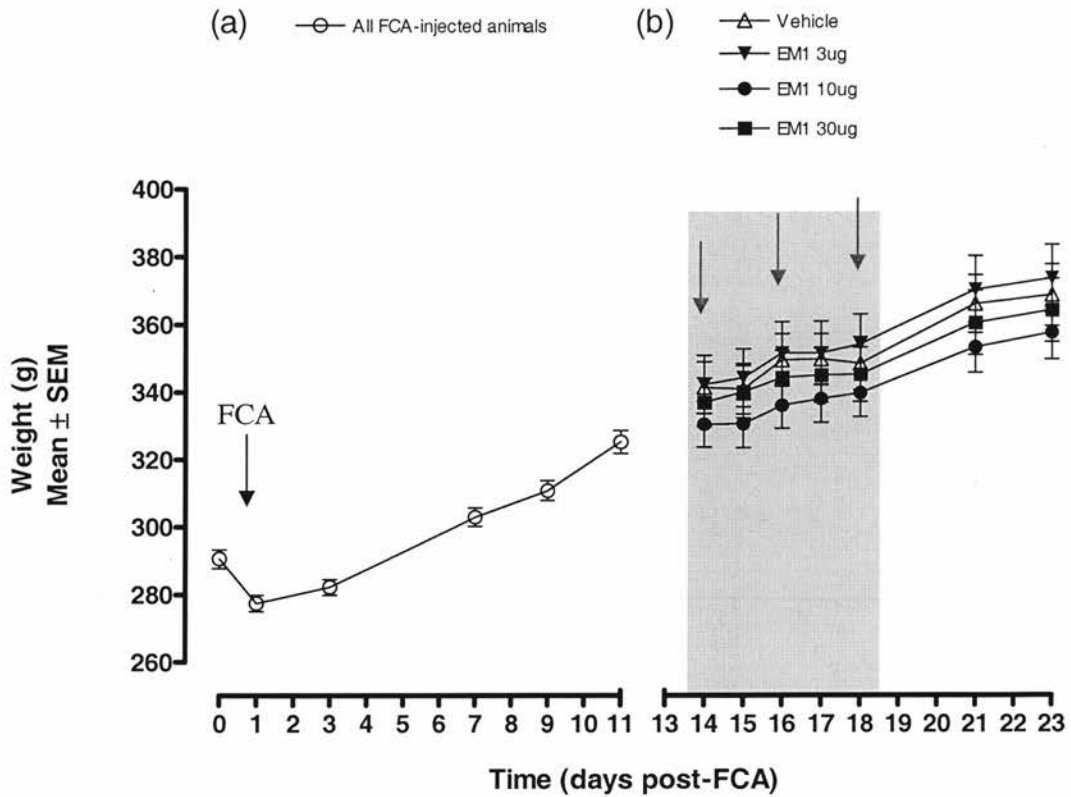


Figure 4.6 Figure illustrating (a) pooled body weights of all animals (n=32, days 0-11) and (b) the same animals in their designated dosing groups for EM1 (n=8, days 14-23) following injection of FCA on day 0. Despite a statistically insignificant decrease in weight from day 0 to day 1 ($P>0.05$, paired t-test, n=32), animals gained weight daily over the duration of the study period. Intra-articular injection of EM1 on days 14, 16 and 18 (indicated by the grey arrows) had no effect on the weight gain of the animals and consequently there was no significant difference in the group mean body weights on days 14 to 23 ($P>0.05$, two-way ANOVA, n=32). The consistent weight gain provides evidence that the animals were not in severe pain or discomfort throughout the studies and were able to continue normal behaviours such as feeding and drinking. The shaded area indicates the drug treatment period.

4.3.1.1 The effect of intra-articular morphine

Baseline weight distribution recordings were made on day 0 and the weight placed through the left and the right hind limb was 53 ± 1 g in both instances, giving a calculated WD ratio of 1.00 ± 0.01 (n=32). Treatment with FCA resulted in the WD ratio on days 4, 7 and 11 post-FCA, decreasing to values significantly lower than the day 0 value ($P<0.05$, one-way ANOVA, n=32, Figure 4.7a).

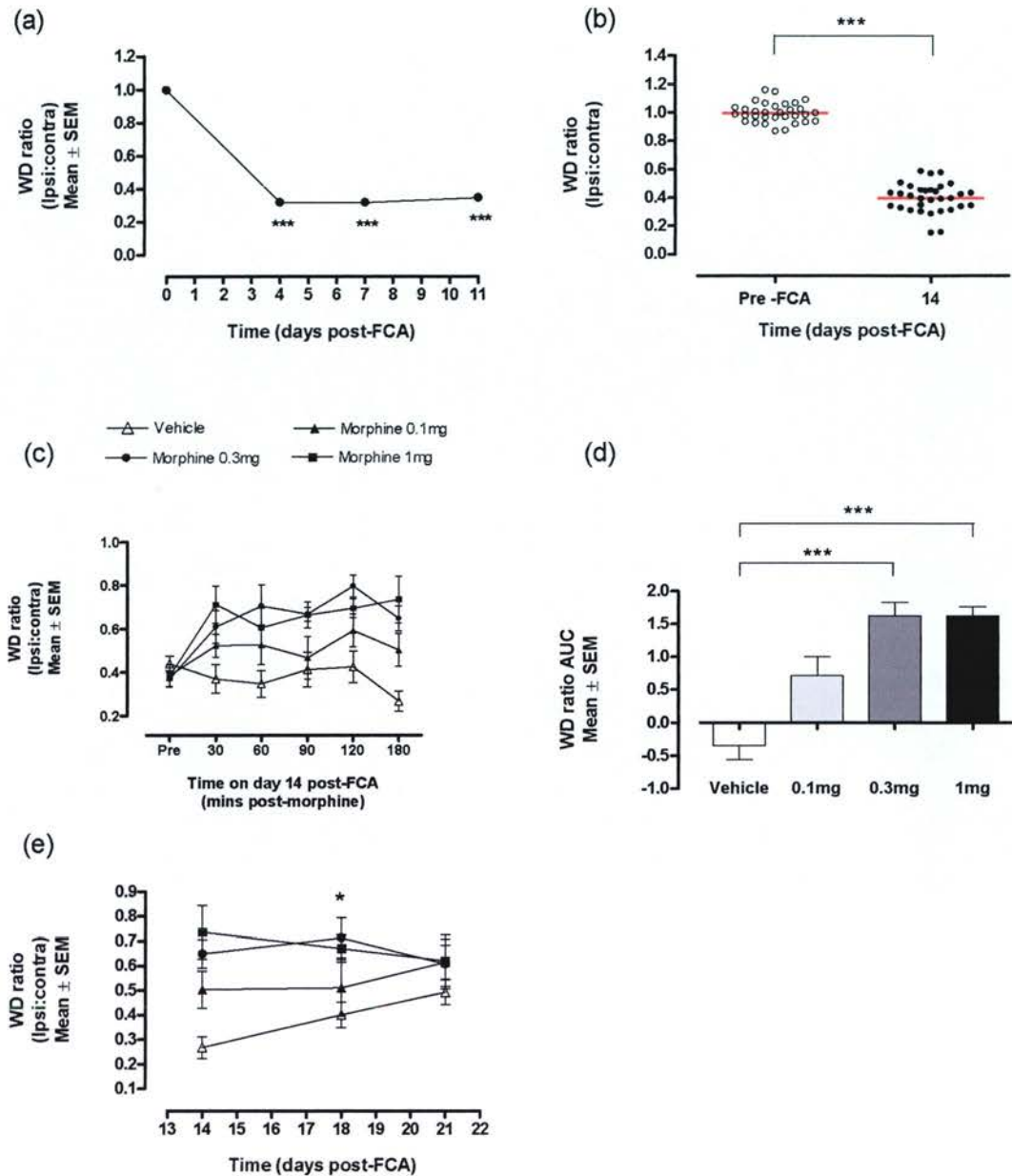


Figure 4.7 Graphs showing the effect of intra-articular FCA and morphine ($1-10\text{mg}\cdot\text{ml}^{-1}$) on the distribution of the animals' body weight between their hind limbs. (a) FCA resulted in a significant decrease in WD ratio on days 4, 7 and 11 post-FCA when compared to day 0 (***) represents $P<0.001$, one-way ANOVA, $n=32$). (b) The WD ratio on day 14, immediately before morphine treatment began, was significantly lower than the pre-FCA value. The mean WD ratios of the morphine 0.1-1mg and vehicle- treated animals on (c) day 14 following the intra-articular administration of the drug and (e) days 14-21 post-FCA. The groups of animals dosed with 0.3 and 1mg morphine had statistically higher WD ratio values than the vehicle treated group on day 18, 4 days after treatment (* $P<0.05$, two-way ANOVA, $n=8$) (d) The WD ratio AUC values during the 3 hours post-morphine on day 14 calculated from the baseline pre-morphine values were analysed to compare the drug-treated groups to the vehicle-treated group. *** represents statistical significance ($P<0.001$) using a one-way ANOVA followed by a Dunn's post-hoc test.

Due to the animals' unwillingness to carry weight through the FCA-injected knee joint, more weight is carried through the leg ipsilateral to the FCA-injected knee ($132 \pm 2\text{g}$) than the FCA-injected leg ($53 \pm 2\text{g}$) and the mean weight distribution ratio on day 14 prior to dosing was 0.39 ± 0.02 ($n=32$, Figure 4.7b).

The effect of administering 0.1, 0.3 and 1mg of morphine or vehicle into the knee joint on the weight distribution of the animals was assessed at half hourly intervals up to 3 hours post-morphine (Figure 4.7c). There was no significant difference in the group mean WD ratios as measured on day 14 prior to the treatment with morphine or vehicle (Table 4.1). Anaesthetic effects from the intra-articular injections were absent 10 minutes after the injection as animals rapidly recovered and exhibited normal cage behaviour such as movement, exploration and feeding. The first behavioural assessment at 30min post-morphine revealed a dose-related reduction in the joint hypersensitivity as animals dosed with 1mg of morphine had a significantly higher WD ratio of 0.71 ± 0.09 compared to the vehicle value of 0.37 ± 0.07 ($P<0.01$, two-way ANOVA, $n=8$, Table 4.1).

From each animal 5 assessments of the WD were made at half hour intervals following administration of morphine or vehicle on day 14. Using each animal's day 14 pre-morphine WD ratio as a basal value, an AUC for the five following assessments was calculated. Results showed significantly higher AUC values for the groups treated with morphine (300 μg and 1mg) compared to the vehicle dosed group. Morphine induced analgesia meaning that animals placed more weight through the limb that was previously hypersensitive, increasing the WD ratio value back towards 1.0 ($P<0.001$, one-way ANOVA, Figure 4.7d).

A WD ratio was calculated from measurements of the weight bearing results made on days 18 and 21 post-FCA (4 and 7 days post-morphine or -vehicle) to observe any long term effect of the intra-articular injection of drug. On day 18 post-FCA there was still a significant difference between the 3 and 10mg.ml⁻¹ morphine dosed groups compared to the

vehicle treated group ($P<0.05$, two-way ANOVA, $n=8$). There was no significant difference between any of the groups on day 21 post-FCA (Figure 4.7e).

Time (minutes)	Vehicle	Morphine 0.1mg	Morphine 0.3mg	Morphine 1mg
Pre-injection	0.44	0.39 (ns)	0.37 (ns)	0.38 (ns)
30	0.37	0.52 (ns)	0.61 (ns)	0.71 (**)
60	0.35	0.53 (ns)	0.71 (**)	0.61 (ns)
90	0.41	0.47 (ns)	0.67 (ns)	0.66 (ns)
120	0.43	0.59 (ns)	0.80 (**)	0.70 (*)
180	0.27	0.50 (ns)	0.65 (***)	0.74 (***)

Table 4.1 The WD ratio values of FCA-injected animals measured on day 14 at the different time point post- morphine or vehicle injection. Brackets illustrate whether there was any significant difference between the morphine groups compared to the vehicle treated group of animals at each time point studied (ns represents no statistical difference, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, two-way ANOVA).

4.3.1.2 The effect of intra-articular EM1

Weight distribution recordings were made on day 0 to provide basal data, with the mean amount of weight placed through the left and right hind limb being 110 ± 2 g and 113 ± 3 g respectively, resulting in a day 0 WD ratio of 1.00 ± 0.02 ($n=32$). Treatment with FCA resulted in the WD ratio on days 1, 3, 7, 9 and 11 being significantly lower than the day 0 value ($P<0.05$, one-way ANOVA, $n=32$, Figure 4.8a). The hypersensitivity is still present on day 11 post-FCA when the animals placed 75 ± 4 g through the FCA-injected limb and 199 ± 3 g through the non-injected limb. This resulted in the day 11 post-FCA WD ratio being significantly lower than the pre-FCA value ($P<0.001$, 0.31 ± 0.02 vs. 1.00 ± 0.02 , Wilcoxon t-test, $n=32$, Figure 4.8b).

The effects of EM1 at 3, 10 and 30 μ g or vehicle into the knee joint on three separate occasions (day 14, 16 and 18 post-FCA) on the weight distribution of the animals was investigated on days 14-23 (Figure 4.8c). There was no significant difference between the mean WD ratios of the four groups as measured on day 11 prior to the dosing (Table 4.2). Measurements made to investigate the effect of the injected drug during the dosing period

revealed that there was a significant increase in the WD ratio of the EM1 30 μ g group on day 14, 21 and 23 post-FCA and the EM1 3 μ g group on day 18 post-FCA when compared to the vehicle group ($P < 0.05$, two-way ANOVA, $n = 8$, Figure 4.8c and Table 4.2). AUC values were calculated for individual animals throughout the duration of the dosing period (day 14–18 post-FCA), using the day 11 values as the basal value. Results showed significantly higher AUC values for the 10 and 30 μ g EM1 dosed groups compared to the vehicle dosed group (* $P < 0.05$, ** $P < 0.01$, one-way ANOVA, Figure 4.8d).

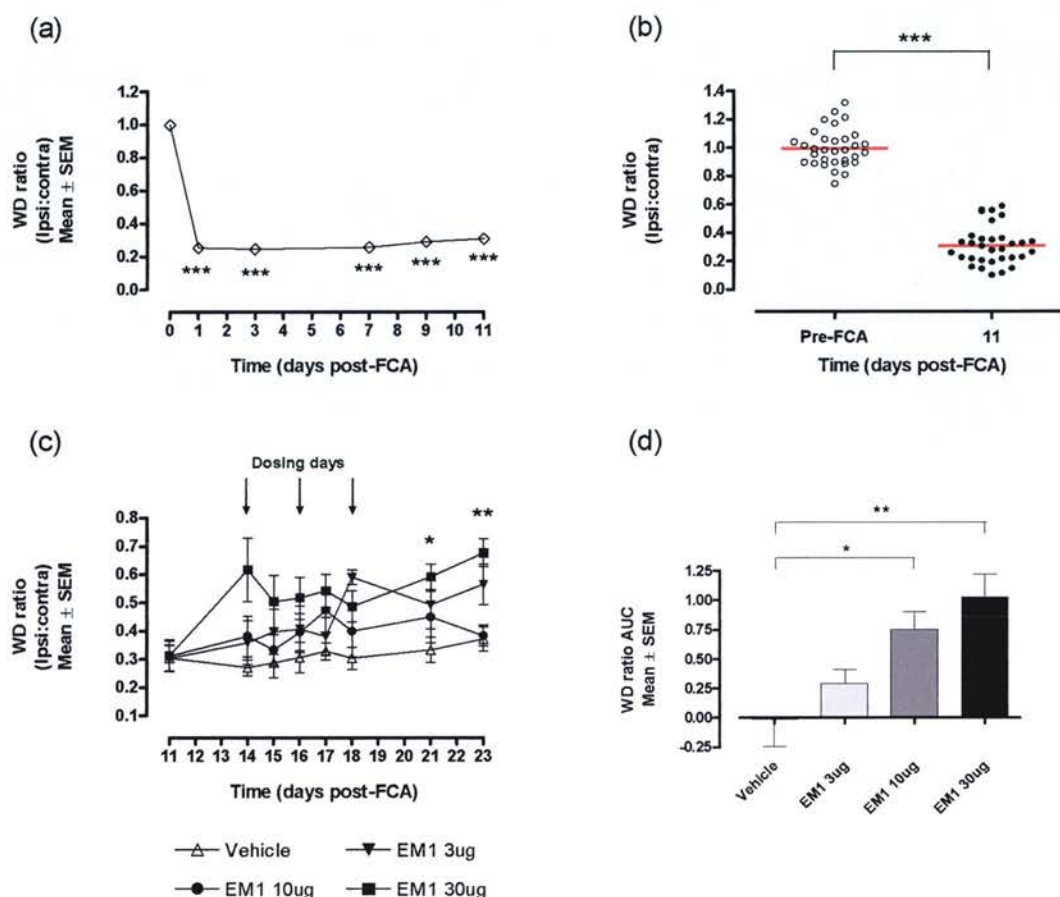


Figure 4.8 Graphs showing that (a) FCA resulted in a significant decrease in WD ratio on days 3, 7, 9 and 11 post-FCA as compared to day 0 values (***) represents $P < 0.001$, one-way ANOVA, $n = 32$). (b) The WD ratio on day 11 was significantly lower than the day 0 value (***) $P < 0.001$, 0.31 ± 0.02 vs. 1.00 ± 0.02 , Wilcoxon t-test, $n = 32$). (c) The mean WD ratios of the EM1 and vehicle-treated animals on day 11–23 post-FCA. The 30 μ g group mean WD ratio was significantly higher than the vehicle group on days 21 and 23 post-FCA. (d) The WD ratio AUC values for the dosing period (day 14–18 post-FCA) were calculated from the basal day 11 values and were analysed to compare the drug-treated groups to the vehicle-treated group. EM1 at 10 and 30 μ g were significantly larger than the vehicle treated group of animals (* $P < 0.05$, ** $P < 0.01$, one-way ANOVA, $n = 8$).

Time (days post-FCA)	Vehicle	EM1 3µg	EM1 10µg	EM1 30µg
11	0.30	0.30 (ns)	0.31 (ns)	0.31 (ns)
14	0.27	0.36 (ns)	0.38 (ns)	0.62 (**)
15	0.29	0.40 (ns)	0.33 (ns)	0.50 (ns)
16	0.31	0.41 (ns)	0.40 (ns)	0.52 (ns)
17	0.33	0.38 (ns)	0.47 (ns)	0.54 (ns)
18	0.30	0.59 (*)	0.40 (ns)	0.49 (ns)
21	0.33	0.49 (ns)	0.45 (ns)	0.59 (*)
23	0.37	0.56 (ns)	0.38 (ns)	0.68 (**)

Table 4.2 The WD ratio values measured on day 11-23 post-FCA, following intra-articular injection of EM1 or vehicle on days 14, 16 and 18. Brackets show whether there was any significant difference between the EM1 groups compared to the vehicle treated group of animals during the dosing period. There was no significant difference between the mean WD ratio of the four groups on day 11, the last measurement before dosing on day 14 (ns represents no statistical difference, * $P < 0.05$, ** $P < 0.01$, two-way ANOVA).

4.3.2 Effect of EM1 and morphine on neural discharge of the MAN

Neural discharge was recorded from 83 afferent fibres in 32 experiments where capsaicin evoked discharge was investigated, and 41 experiments from a total of 93 afferent fibres where mechanically evoked discharge was investigated. Afferents were classed as polymodal C-fibres or mechanosensitive A δ -fibres based on their conduction velocity, sensitivity to capsaicin and response to noxious mechanical stimulation as previously described (see section 3.3.2). The few fibres that did not respond to capsaicin or noxious mechanical stimulation were not considered to be nociceptive and were not investigated further in these studies.

In experiments which investigated capsaicin evoked firing of the MAN, 16 out of 83 fibres (19%) were mechanosensitive, but did not respond to capsaicin and were classed as A δ -fibres, whilst the other 67 fibres (81%) were responsive to both capsaicin and mechanical stimulation and were identified as being polymodal nociceptors. In the mechanically evoked firing of the MAN experiments 28 out of 93 fibres (30%) were identified as

mechanosensitive A δ -fibres and 65 out of 93 fibres (70%) were identified as polymodal C-fibres responding to both noxious mechanical stimulation and capsaicin (see Table 4.3).

	Capsaicin evoked Firing (n=32)	Mechanically evoked firing (n=41)
Aδ-fibres	16/83 (19%)	28/93 (30%)
C-fibres	67/83 (81%)	65/93 (70%)

Table 4.3 Proportions of fibre types of fibre types innervating the knee joints investigated in the capsaicin and mechanically evoked firing of the MAN experiments.

4.3.2.1 Blood pressure and heart rate

The effect of capsaicin, morphine and EM1 on the arterial blood pressure (B.P.) and heart rate of the anaesthetised animal was recorded to detect pharmacological changes evoked by the drugs administered (Table 4.4). The mean control B.P. in experiments prior to injection of any drug was 119 ± 3 mmHg and the heart rate was 394 ± 12 bpm (n=37).

		Capsaicin	Morphine	EM1
Control	Sys B.P.	153	131	139
	Dias B.P.	117	100	106
	Mean B.P.	129	110	117
	Heart Rate	409	418	388
Primary Effect	Sys B.P.	131	138	108
	Dias B.P.	108	104	68
	Mean B.P.	116	116	81
	Heart Rate	392	406	332
	Delta D.P.	3	5	-36
	Delta H.R.	-17	-7	-56
Secondary effect	Sys B.P.	160	111	-
	Dias B.P.	120	77	-
	Mean B.P.	133	88	-
	Heart Rate	397	372	-
	Delta B.P.	14	-21	-
	Delta H.R.	-13	-46	-

Table 4.4 Table summarising the effects of 1 μ g capsaicin, 300 μ g morphine and 100 μ g EM1 on the mean blood pressure and heart rate of the anaesthetised animals during the neural recordings. Capsaicin and morphine had a two phase effect on the blood pressure, whereas EM1 only had a single stage effect. All drugs had the effect of decreasing the heart rate of the animals. Capsaicin caused an initial drop in heart rate followed by a secondary phase increase. Morphine caused an initial increase, with the secondary effect to be a decrease in blood pressure. Administration of EM1 resulted in a decrease in blood pressure.

Injection of capsaicin resulted in a two phase effect on the B.P. with a primary decrease of $3 \pm 10\text{mmHg}$, and a slower secondary increase of $14 \pm 10\text{mmHg}$ from the initial control value. The heart rate decreased to $392 \pm 12\text{bpm}$ and $397 \pm 10\text{bpm}$ at the peaks of these two responses respectively when compared to control values of $409 \pm 9\text{bpm}$.

Morphine also resulted in the B.P. to be changed in two distinct phases. The initial response saw a mean increase in the B.P. of $5 \pm 2\text{mmHg}$ followed by a secondary response of a mean decrease of $21 \pm 4\text{mmHg}$. The heart rate of the animals slowed throughout both stages, decreasing by $7 \pm 6\text{bpm}$ and $46 \pm 8\text{bpm}$ when compared to control respectively.

Injection of EM1 resulted in a single phase effect on the B.P. with the mean control of $117 \pm 7\text{mmHg}$ dropping $36 \pm 4\text{mmHg}$ to a post-drug mean B.P. of $81 \pm 5\text{mmHg}$. The heart rate fell by $56 \pm 10\text{bpm}$ during this post-drug period.

4.3.3 Capsaicin-evoked neural discharge

4.3.3.1 Repeat capsaicin and vehicle control experiments

Repeat injections of capsaicin evoked action potential discharge of the fibres in the MAN. In both naïve ($n=5$, fibres=18) and FCA-injected ($n=6$, fibres=12) animals capsaicin was injected on five separate occasions at intervals of 45 minutes between each test. Consistent discharge frequencies were observed with no sensitisation or desensitisation of the nerve. There was no significant difference between the mean discharge frequency (impulses.s^{-1}) of all the tests performed in both naïve and FCA-injected animals ($P>0.05$, one-way ANOVA, Figure 4.9a and 4.9b respectively). The mean discharge frequencies evoked in these experiments ranged from 14-21 impulses.s^{-1} which is in keeping with the range of 13-22 impulses.s^{-1} obtained from all the experiments involving capsaicin as described in section 3.3.2.2. Injection of vehicle (saline) at 70 minutes as a control for opioid agonist injections, in between the second and third capsaicin test had no effect on the

capsaicin response. There was no significant difference between the mean discharge frequencies of the first or second capsaicin test at 0 and 45 minutes, confirming that these tests can be used as pre-drug baseline values ($P>0.05$, Wilcoxon t-test, Figure 4.9a and 4.9b).

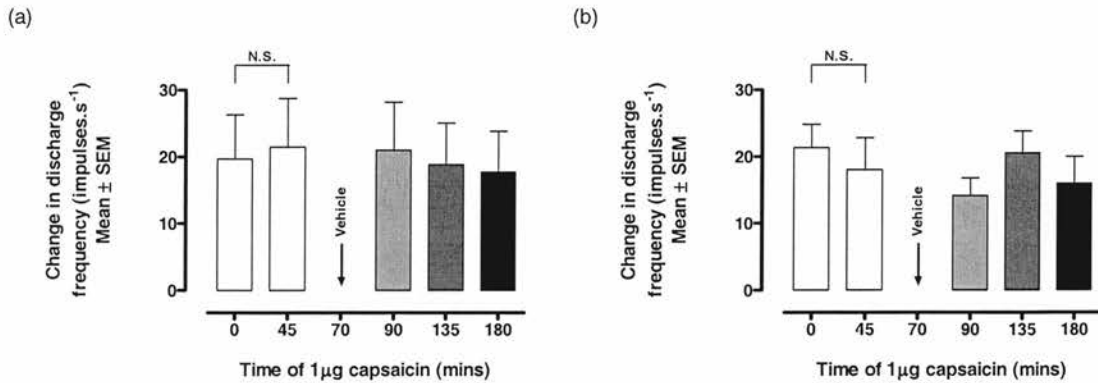


Figure 4.9 Capsaicin evoked neural discharge of fibres innervating (a) the naïve ($n=5$, fibres=18) and (b) the FCA-injected ($n=6$, fibres=12) knee joint before and after vehicle (saline) administration. There was no significant difference in the neural discharge frequency at any time point following repeat capsaicin injections ($P>0.05$, one-way ANOVA).

4.3.3.2 Effect of morphine on capsaicin evoked discharge

In both naïve ($n=5$, fibres=12) and FCA-injected ($n=6$, fibres=12) animals, two doses of capsaicin were injected 45 minutes apart prior to the test drug morphine (300 µg). There was no significant difference between the mean discharge frequencies of the first and second capsaicin tests in either case ($P>0.05$, Wilcoxon t-test, Figure 4.10a and 4.10b). In naïve animals the injection of morphine did not effect the subsequent responses to capsaicin at 110, 155 and 200 minutes, in comparison with the first two tests ($P>0.05$, one-way ANOVA, Figure 4.10a). In FCA-injected animals morphine resulted in a significant reduction in the mean discharge frequency evoked by the capsaicin test performed at 200 minutes when compared to the basal test at 0 minutes ($P<0.01$, one-way ANOVA, Figure 4.10b).

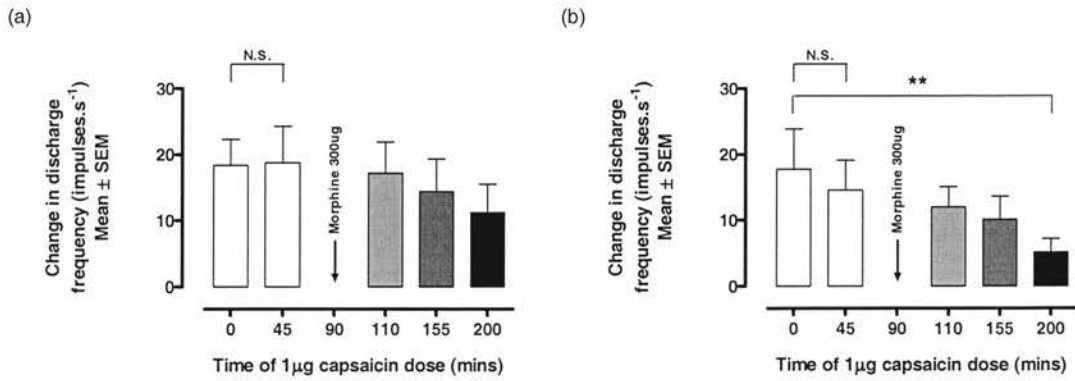


Figure 4.10 Graphs showing capsaicin (1 µg) evoked neural discharge of fibres innervating (a) naïve (n=5, fibres=12) and (b) FCA-injected (n=6, fibres=12) knee joint in two tests prior to and three tests following injection of 300 µg morphine. (a) There was no significant difference in the neural discharge frequency at any time point in naïve animals ($P > 0.05$, one-way ANOVA). (b) Morphine (300 µg) caused a significant reduction in the capsaicin evoked neural discharge at 200 minutes when compared to 0 minutes ($P < 0.01$, one-way ANOVA).

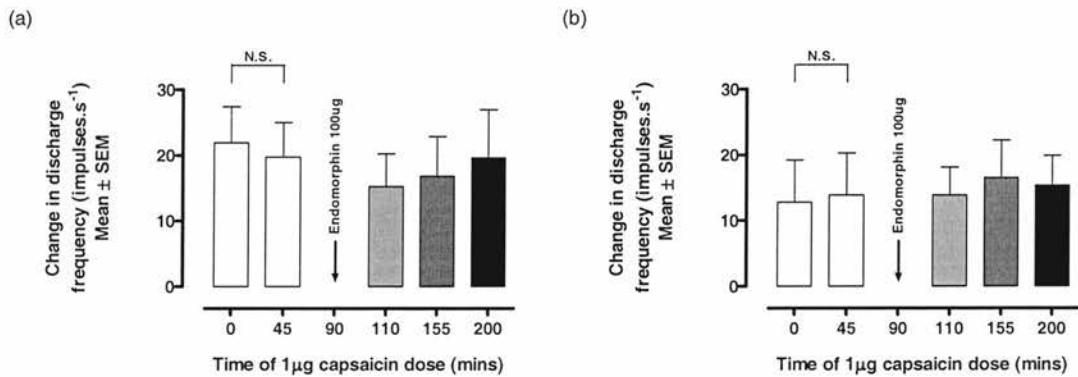


Figure 4.11 Capsaicin (1 µg) evoked neural discharge of fibres innervating (a) naïve (n=6, fibres=15) and (b) FCA-injected (n=5, fibres=17) knee joint before and after administration of 100 µg of EM1. There was no significant difference in the neural discharge frequency at any time point following repeat capsaicin injections ($P > 0.05$, one-way ANOVA).

4.3.3.3 Effect of EM1 on capsaicin evoked discharge

The two initial injections of capsaicin in both naïve (n=6, fibres=15) and FCA-injected (n=5, fibres=17) animals did not differ significantly from each other ($P > 0.05$, Wilcoxon t-test, Figure 4.11a and 4.11b). Injection of EM1 had no effect on the mean evoked discharge frequency in both naïve and FCA-injected animals. There was no

significant difference in the pre-injection capsaicin tests compared to any of the three post-injection capsaicin tests ($P>0.05$, one-way ANOVA, Figure 4.11).

4.3.4 Mechanically-evoked neural discharge

4.3.4.1 Repeat mechanical and vehicle control experiments

In both naïve ($n=6$, fibres=13) and FCA-injected ($n=5$, fibres=14) animals, the von Frey filaments, were applied to the surface of the tissue surrounding the knee joint capsule for 10s with a rest period of 10s in between each filament. These tests were repeated ten times at intervals of 20 minutes between each set of three applications. Consistent discharge frequencies were observed in both naïve and FCA-injected animals following repeat applications of the 1, 7 and 21g (14, 98 and 255mN) filaments over the course of the experiments (Figure 4.12a and 4.12b). There were no significant differences between the mean discharge frequency (impulses. s^{-1}) evoked by the filaments, in naïve or FCA-injected animals, when each filament was analysed independently of the other two ($P>0.05$, one-way ANOVA, Figure 4.12). Moreover the linear regression analysis for each filament in both naïve and FCA-injected animals, was not significantly different from zero suggesting the trend is for the nerve to be neither sensitised nor desensitised by repeat von Frey filament applications. Injection of vehicle (saline) at 95 minutes had no effect on the discharge frequencies evoked by any of the individual filaments in the following tests. There was no significant difference between the mean discharge frequencies evoked by the 1, 7 and 21g filaments when comparing the pre-saline tests with the post-saline tests ($P>0.05$, one-way ANOVA, Figure 4.12b and 4.12d).

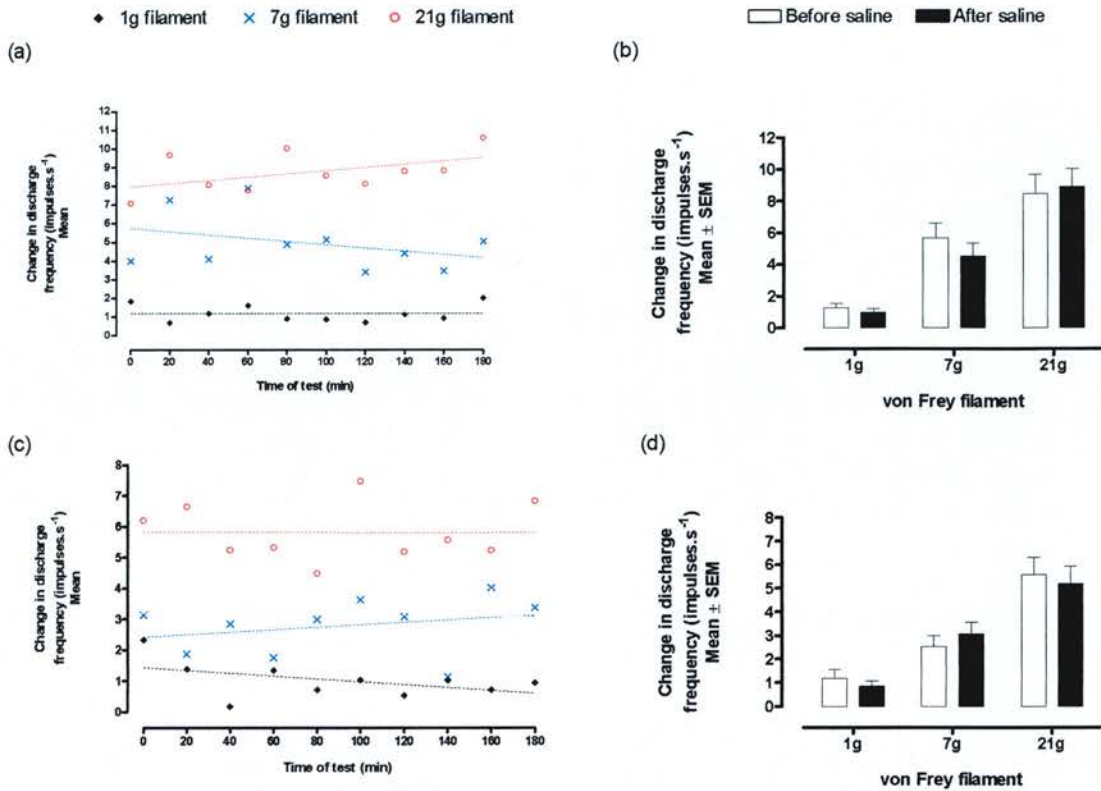


Figure 4.12 Graphs showing the mean change in the frequency of neural discharge above basal activity in (a) and (b) naïve and (c) and (d) FCA-injected animals evoked by a 10s stimulation with a 1, 7 and 21g von Frey filament. (a) and (c) Repeat applications of the 1, 7 and 21g filaments 20 minutes apart produced consistent mean discharge frequencies over the course of the experiment. There was no significant difference in the mean discharge frequencies of the 1, 7 and 21g filaments over time when analysing the filaments individually ($P > 0.05$, one-way ANOVA). Moreover the angle of the linear regression slope for each filament, in both naïve and FCA-injected animals, is not significantly different from zero ($P > 0.05$, linear regression). Injection of saline at 95 minutes had no effect on the neural discharge frequency evoked by the von Frey filaments in both (b) naïve and (d) FCA-injected animals. There was no significant difference in the mean change in discharge frequency evoked when comparing the pre-saline 1, 7 and 21g values with the corresponding post-saline values ($P > 0.05$, one-way ANOVA).

4.3.4.2 Effect of EM1 and morphine on mechanically evoked discharge

In naïve animals, morphine caused a significant reduction in the mean discharge frequency evoked by the 21g filament when comparing the five pre-opioid tests with the five post-opioid tests ($P < 0.05$, one-way ANOVA, $n=6$, fibres=14, Figure 4.13a). There was no significant difference however, between the mean discharge frequencies evoked by the 1 and 7g filament pre- and post-morphine ($P > 0.05$, one-way ANOVA, Figure 4.13a). Injection of

EM1 in naïve animals, had no effect on the mean discharge frequencies evoked by any of the von Frey filaments studied when comparing the tests pre- and post-EM1 ($P>0.05$, one-way ANOVA, $n=6$, fibres=13, Figure 4.13b).

In FCA-injected animals injection of morphine (300µg) resulted in a significant decrease in the mean discharge frequency evoked by the 21g filament, when comparing the post-drug evoked firing to the pre-drug evoked firing ($P<0.05$, one-way ANOVA, $n=5$, fibres=11, Figure 4.13c). Injection of EM1 (100µg) resulted in significant decreases in the post-drug mean change in discharge frequency compared to pre-drug with all the filaments investigated (* $P<0.05$, *** $P<0.001$, one-way ANOVA, $n=5$, fibres=10, Figure 4.13d).

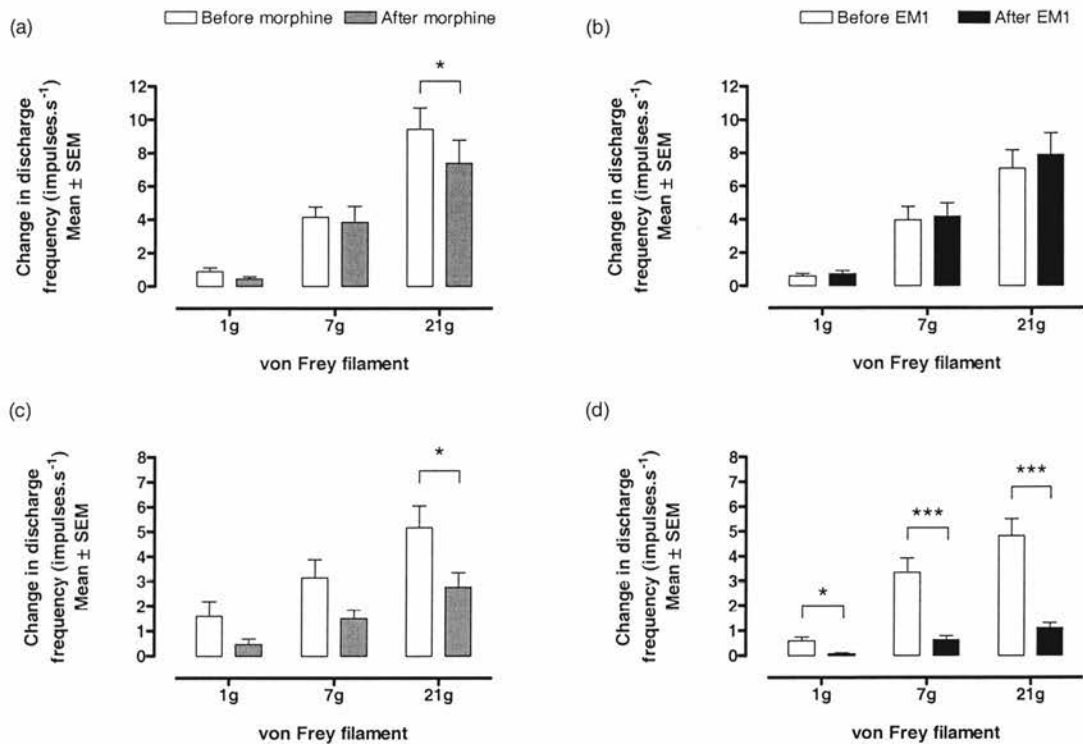


Figure 4.13 Graphs showing the effect of morphine (300µg) and EM1 (100µg) on von Frey filament evoked firing in (a) and (b) naïve and (c) and (d) FCA-injected animals. The mean change in the frequency of neural discharge from background activity in naïve animals evoked by application of the 1, 7 and 21g von Frey filament before and after injection of (a) morphine ($n=6$, fibres=14) and (b) EM1 ($n=6$, fibres=13). In naïve animals morphine had no effect on the neural discharge frequency evoked by the 1 and 7g von Frey filaments, but significantly reduced the mean discharge frequency evoked by the 21g filament compared to the pre-morphine value ($P<0.05$, one-way ANOVA, $n=6$). In naïve animals injection of EM1 had no effect on the change in mean discharge frequency evoked by any of the filaments pre- and post-EM1 ($P>0.05$, one-way ANOVA, $n=6$). The mean neural discharge frequency in FCA-injected animals evoked by application of the 1, 7 and 21g von Frey filament before and after injection of (c) morphine ($n=5$, fibres=11) and (d) EM1 ($n=5$, fibres=10). Significant attenuation of the mean change in neural discharge from on-going discharge was observed following injection of morphine and EM1. Following injection of morphine the mean discharge evoked by the 21g filament was significantly lower than the mean discharge evoked from the corresponding pre-morphine tests ($P<0.05$, one-way ANOVA, $n=5$). Injection of EM1 resulted in the mean discharge evoked by all three filaments being significantly reduced when compared to their corresponding pre-EM1 values (* $P<0.05$, *** $P<0.001$, one-way ANOVA, $n=5$).

4.3.4.3 Naloxone

In FCA-injected animals pre-treatment with naloxone 180 seconds before the injection of morphine or EM1 prevented the opioid-induced reduction in discharge frequencies evoked by both morphine and EM1 described in the previous section. There was no significant difference between the pre and post-drug mean neural discharge frequencies

evoked by the 1, 7 and 21g von Frey filament following injection of naloxone and morphine ($P>0.05$, one-way ANOVA, $n=4$, fibres=9, Figure 4.14a) and naloxone and EM1 ($P>0.05$, one-way ANOVA, $n=4$, fibres=9, Figure 4.14b).

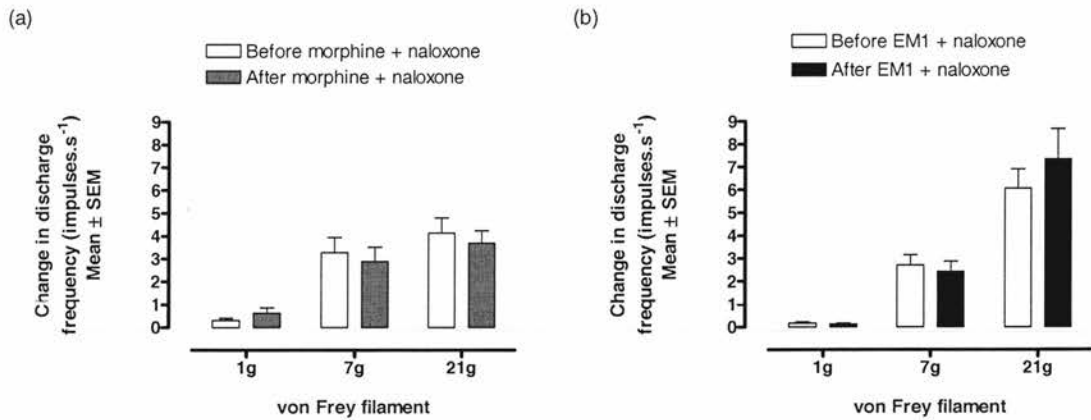


Figure 4.14 Effect of the opioid antagonist naloxone (500µg) on the mean neural discharge frequency in FCA-injected animals evoked by application of the 1, 7 and 21g von Frey filament before and after injection of (a) morphine (300µg, $n=4$, fibres=9) and (b) EM1 (100µg $n=4$, fibres=9) where naloxone was injected 180 seconds before the test drugs. Injection of morphine and EM1, following pre-treatment with naloxone, resulted in there being no significant difference between pre- and post-drug discharge frequencies at all 3 von Frey filaments ($P>0.05$, one-way ANOVA).

4.4 Discussion

The main finding from these studies is that peripherally administered morphine and EM1 attenuated FCA-induced joint hypersensitivity, as measured by the behavioural shift in the amount of weight placed through the inflamed knee. Furthermore morphine and EM1 also reduced the nociceptive discharge evoked mechanically by von Frey filament stimulation of the knee joint capsule in electrophysiological recordings from the MAN. These effects were evident in animals 14-23 days post-FCA injection in the behavioural experiments and 14-28 days post-FCA in the *in vivo* electrophysiology experiments and were abolished by the pre-treatment of the opioid receptor antagonist naloxone. This evidence supports the hypothesis that functional opioid receptors are present on a population of

nociceptive afferents innervating the knee joint of rats at a more advanced stage of the FCA-inflamed knee joint model than previously investigated (Li et al. 2005).

4.4.1 Discussion of behaviour results

Intra-articular administration of morphine and EM1 reduced FCA-induced joint hypersensitivity. This was measured using the incapacitance tester, which revealed an increase in WD ratio towards the normal basal value of 1.0. Single doses of morphine (0.3 and 1mg) given directly into the knee joint significantly attenuated the FCA-induced hypersensitivity at various times on the day of dosing in comparison with vehicle, an effect that was still present 4 days after the initial dose. Similarly, EM1 injected directly into the knee joint at a dose of $300\mu\text{g.ml}^{-1}$ produced a significant reversal of the FCA-induced joint hypersensitivity back towards a normal weight distribution, half an hour after the initial injection on day 14. Furthermore, the overall effect of the three injections of the different doses of EM1 given was a significant attenuation of the FCA-induced joint hypersensitivity in the 10 and $30\mu\text{g}$ dosed animals, compared to the vehicle dosed animals over the five day period. This effect was still present on days 21 and 23 post-FCA and occurred in the highest dose. These results are in accord with the clinical findings that a single intra-articular injection of morphine provided a long lasting analgesia of up to 5 days post injection in patients suffering from arthritis in the knee joint (Likar et al. 1997; Stein et al. 1999).

Morphine and EM1 were injected directly into the FCA-injected knee joint under brief halothane anaesthesia, a route of administration that has been previously used clinically (Christophidis and Huskisson 1982). The mechanism of action of general anaesthetics in mammals is not fully understood and halothane has been implicated in sensitising cutaneous primary afferent nociceptors to heat stimuli in the anaesthetised monkey, a process that was fully reversible (Campbell et al. 1984; Franks and Lieb 1994). In the present study animals were allowed a minimum 30 minute recovery period after the injection prior to performing

the behavioural assessments which allowed any anaesthetic effects to wear off. It is probable that the low concentrations of halothane used and the short period of time the animals were anaesthetised ensured that the observed analgesic effects were not due to the brief anaesthesia, but were the result of morphine or EM1. Moreover, as the WD ratio calculated from the incapacitance tester is dependent on the amount of weight carried by both hind limbs, it would be reasonable to consider the un-injected limb as an internal control for any effect of the general anaesthesia, as any effect present in the injected leg would also be present in the un-injected leg.

The first 30 μ g dose of EM1 caused a statistically significant increase in the group mean WD ratio from the pre-drug, a change that was comparable with the increase observed following the first dose of morphine at 1mg. The 30 μ g dose of EM1 injected into the knee joint was equivalent to an estimated maximum concentration of 245 μ M and produced a similar analgesic efficacy to the higher dose of morphine, which was equivalent to an estimated maximum concentration of 6.6mM. In a previous behavioural study the ED₅₀ of EM1 dosed directly into the spinal cord of mice was half that of the morphine dosed in the same study (Zadina et al. 1997). The reason for the higher efficacy of EM1 in both the Zadina study and the present study is due to the fact that EM1 has a higher affinity and specificity for the μ -opioid receptor, therefore causing the potent analgesic effect associated with μ -opioid receptor agonists (Zadina et al. 1997). To examine whether the analgesic effects observed in the present behavioural study were due to μ -opioid receptor activation, a follow-up behavioural study could be conducted in which EM1 is dosed in the same injection along with a μ -opioid receptor selective antagonist such as CTOP, the conformationally constrained synthetic cyclic peptide related to somatostatin (Pelton et al. 1986).

4.4.2 Discussion of capsaicin-evoked neural discharge results

Close intra-arterial injection of capsaicin resulted in fast onset nociceptive discharge activated through the ligand-gated ion channel TRPV1 (Caterina et al. 1997). Activation of the fibres in response to capsaicin was similar in both naïve and FCA-injected animals as discussed in section 3.3.2.2., with repeat administration producing consistent levels of neural discharge due to the 45 minute rest period in between each dose. Desensitisation of C-fibres has been previously reported in a study where a capsaicin solution was applied topically to the skin of rats (Lynn et al. 1992). Furthermore capsaicin administered to neonates can be neurotoxic, resulting in the destruction of the majority (95%) of nociceptors in rats (Fitzgerald 1983; Karai et al. 2004). It was important in the present study that capsaicin should not desensitize the TRPV1 receptor as strong evidence exists that this is the analgesic mechanism of action of capsaicin based topical creams used by patients suffering from painful neuropathies (Szallasi 2002).

Neither morphine nor EM1 had any affect on the capsaicin-evoked increase in nociceptive discharge in naïve animals. The only significant effect of either drug in this set of experiments was observed on the final test in FCA-injected animals following close arterial delivery of morphine. Any effect of morphine or EM1 would be expected to occur earlier than 110 minutes after the injection, since the μ -opioid receptor is G-protein coupled and an analgesic effect mediated through an immediate secondary messenger system can occur as soon as 10 minutes after the drug is given (Ikeda et al. 2000). An alternative hypothesis for the significant reduction in capsaicin evoked neural discharge observed at this late time point is that morphine-induced vasoconstriction of blood vessels supplying the knee joint and the MAN microcirculation (Schaafsma et al. 1997) could lead to less of the exogenous capsaicin circulating around the MAN.

4.4.3 Discussion of mechanically-evoked neural discharge results

Graded von Frey filament stimulation is a quantitative method of investigating mechanical allodynia and hyperalgesia in behavioural and electrophysiology studies in rats, already discussed in section 3.4. As in the capsaicin work described above repeat mechanical stimulation produced a consistent neural discharge in response to each of the three von Frey filaments over the duration of the 180 minute experiments in both naïve and FCA-injected animals. In the naïve animals, morphine reduced the discharge frequency evoked by the 21g filament and had no effect in response to the 1 and 7g applications when pre- and post-drug responses were matched. There was no significant difference between the pre- and post-drug neural response to any of the filaments following the injection EM1. As the 7 and 21g filament are considered to be nociceptive in this experimental paradigm, it could be concluded from the results presented here that the dose of EM1 chosen was not high enough to produce an antinociceptive effect of the noxious mechanical stimulation in naïve animals. However, the cardiovascular effects associated with both drugs show that the doses of morphine and EM1 used were sufficient to cause a significant decrease in the B.P and heart rate of the animals at the time of injection, and any increase in dose may lead to a potentially fatal decrease in blood pressure and was therefore not risked at this time.

In FCA-injected animals both morphine and EM1, given at the same dose as used in naïve animals, resulted in an observable reduction in the mechanically evoked discharge by the 1, 7 or 21g filament. Morphine significantly reduced the frequency of firing evoked by the 21g filament, and EM1 also caused a significant reduction in the frequency of firing of the nerve evoked by all 3 filaments when comparing pre- and post-responses. The antinociceptive effect of these opioids in the arthritic animals is clearly mediated through a μ -opioid receptor mechanism, because pre-treatment with naloxone abolished the opioid-induced reduction in neural discharge. The difference in antinociceptive potency observed between naïve and FCA-injected animals could be due to a number of FCA-induced

inflammatory mechanisms. FCA has been shown to demyelinate peripheral A δ -fibres (Mizisin et al. 1987) and this process could lead to opioid receptors on the primary afferent nerve axons becoming exposed to the locally administered drugs that are not normally accessible in the naïve animals because the intact perineural membrane acts as a barrier. Perineural damage following inflammation has been reported to occur within 12 hours of the inflammatory insult as a result of a low local pH (Antonijevic et al. 1995).

Inflammation has also been associated with an up-regulation of the μ -opioid receptor in DRG of small diameter primary afferent nerves C-fibres, with axonal transport of the receptors to the periphery being dependent on neuronal conduction (Puehler et al. 2004). Evidence also exists suggesting that inflammation increases μ -opioid receptor binding affinity and enhances the efficacy of full and partial μ -opioid receptor agonists in G-protein coupling (Zollner et al. 2003).

4.4.4 Summary

The results of the present study, together with publications from Stein's research group and collaborators on mechanisms of peripherally-mediated opioid analgesia (Stein 1991; Schafer et al. 1995; Stein 1995; Machelska et al. 1999; Stein et al. 2003; Zollner et al. 2003) support the hypothesis that functional opioid receptors are present on peripheral nociceptive afferent nerve fibres innervating the inflamed rat knee joint. A typical value for the proportion of non-inflamed unmyelinated peripheral sensory nerve axons expressing the μ -opioid receptor is 29% (Coggeshall et al. 1997). It is reasonable to hypothesise that inflammation triggers an increase in the production of μ -opioid receptors in the DRG of nociceptors, and these opioid receptors are transported to the periphery as part of the inflammatory response. This would certainly help explain the clinical analgesic action of peripherally administered opioid agonists in the management of inflammatory joint pain.

The results presented in this chapter do not concur with studies reporting that the supply of the opioid receptors from the DRG to the periphery is such that it is unable to maintain a functional population of receptors as they are down-regulated as a consequence of inflammation (McDougall et al. 2004a; b; Li et al. 2005). As the models of inflammation used in those studies and the present study are the same, utilising an intra-articular injection of FCA, it is puzzling that the results produced appear to contradict each other. An explanation may be that a proportion of the functional opioid receptors are down-regulated at the time points studied in the McDougall group publications, but some functional receptors are still present, all be it in reduced numbers. The doses administered in these studies may not have been sufficiently high enough to elicit a response in the remaining functional receptors, leading the group to conclude that they are completely absent at that time. In order to further understand and evaluate the full therapeutic potential of opiate receptor agonists in the treatment of chronic inflammatory pain it will be necessary to perform similar pre-clinical studies in chronic stages of other inflammatory pain models that exist. Moreover investigations performed long after the initial inflammatory insult may be more comparable to the established chronic pain observed in the clinic.

In conclusion, the present study supports the hypothesis that peripherally administered or restricted opioid receptor agonists may play an important role in long term pain management, with the benefit of avoiding the undesirable centrally mediated side-effects seen following systemic opioid administration.

Chapter 5

Development and validation of the pressure application measurement (PAM) device

5.1 Introduction

Chronic joint pain is the most common symptom described by patients suffering from musculoskeletal conditions such as OA (Tallon et al. 2000; Dieppe and Lohmander 2005). The prevalence of both OA and RA is high within the UK adult population, with estimated incidences of 12.5% (Bedson et al. 2005) and 0.8% (Symmons 2002) respectively. Patients suffering from RA generally report to health care professionals that improving the joint pain associated with their condition as having the highest priority (Heiberg and Kvien 2002; Heiberg et al. 2005).

There have been many studies searching for a single, reliable, and clinically convenient measure of pain in inflamed joints in patients with RA (Lansbury and Haut 1956; Mainland 1967). Measuring joint pain in the clinic is often performed by the patient in the form of a 'subjective' self-assessed questionnaire (Gooberman-Hill et al. 2007). Several of these questionnaires, such as the visual analogue scale, have been developed to provide a score of pain status and functionality of the affected joint (Melzack 1975; Bellamy et al. 1988; Roos et al. 1998; Anderson 2001; Adamson et al. 2004). Responses to these standardised questions can lead to high volumes of low cost, quantitative data that can be easily used to monitor whether a patient's condition improves, is maintained or worsens over time, however are not usable in pre-clinical animal models.

As an alternative to patient self-assessments, instruments and physical examinations have regularly been used to directly assess joint tenderness (Sokka 2003). The Ritchie articular index (Ritchie et al. 1968) is an examination that involves firmly squeezing the knee joint and then grading the tenderness of the joint based on the patients reaction using a four point scale (Table 5.1).

Score	0	1	2	3
Patients reaction	Not tender	Tender	Tender and winced	Tender, winced and withdrew

Table 5.1 A summary table detailing the Ritchie index used for assessing joint hypersensitivity in OA and RA patients. A score of 0-3 is awarded depending on the patients' reaction to squeezing of the joint.

This method is a generally accepted measure of assessing joint pain, but it still remains subjective and therefore potentially unreliable. The algometer and sphygmomanometer were the first objective instruments designed to measure joint tenderness by applying a graded pressure across the joint (Mandel 1956). Currently used in the clinic, the dolorimeter is an instrument that measures joint tenderness in much the same way (Langley et al. 1983). A gradually increasing localised pressure is applied perpendicularly across the affected joint using this device until the patient reports tenderness or pain, at which point a sensitivity score is recorded. In a drug withdrawal study the dolorimeter was able to detect subtle changes in joint tenderness whereas the Ritchie articular index was not (Langley et al. 1983), suggesting that by removing subjectivity from the test improves the accuracy of the final outcome.

There is demand in the clinic to successfully manage long-term chronic joint pain (Ashburn and Staats 1999; Rubin 2005), and as a result several rodent models of chronic inflammatory joint pain have been developed as discussed in the introduction to this thesis. These models are commonly used as pre-clinical screens to measure the efficacy of novel analgesics which aim to improve on the treatments currently available. In order to do this successfully, it is essential that results and assessments from animal models in pre-clinical tests accurately reflect observations made in the clinic. Animal self-assessments are obviously not possible, and with the lack of verbal communication, several behavioural readouts have been developed which can be used to assess joint sensitivity in rodents in a reliable and ethical manner.

Historically thermal, mechanical and electrical stimuli have been employed to determine nociceptive thresholds in animals and to date several different behavioural readouts have been used (Le Bars et al. 2001). For instance, the tail-flick test to heat involves focusing a beam of light on the tip of the tail and the latency of tail withdrawal is measured (Stein et al. 1988; Sluka and Westlund 1993). The Randall and Selitto analgesy-meter is operated and an increasing mechanical force is applied to the dorsal surface of the foot of rodents until the weight becomes noxious and the animal withdraws from the apparatus as a reflex (Randall and Selitto 1957; Attal et al. 1988). Behavioural readouts designed to assess gait analysis (Clarke et al. 1997; Hamers et al. 2001; Vrinten and Hamers 2003) and paw elevation time on a rotating cylinder (Tonussi and Ferreira 1992) measure pain indirectly in the form of the mobility of the whole animal. All of these techniques are limited by the fact that they cannot be applied directly to the knee to assess localised joint pain.

A patient with knee joint pain will guard the affected joint by shifting their weight to the other leg, although in RA if both joints are affected then this may not be evident. The incapacitance tester is a commonly used behavioural evaluation that provides an assessment of the hind limb weight distribution in rodents that correlates with the clinical assessment of pain on standing (Schott et al. 1994; Clayton et al. 1997). The apparatus (described in section 2.2.1.) measures the amount of weight the animal places through each hind limb over a three second period. Under normal conditions naïve rodents will distribute their weight evenly across both hind limbs (Kobayashi et al. 2003). Animals with a unilateral joint hypersensitivity alter their resting stance to place less weight on the affected limb. Following treatment with a range of clinically effective gold standard analgesics this uneven stance can be reversed back towards an equal weight distribution (Wilson et al. 2006).

Calibrated forceps housing force transducers have been developed to assess paw limb withdrawal thresholds in behavioural studies, and are often used in electrophysiological experiments to evoke a measureable noxious stimulus (Yu et al. 2002; Luis-Delgado et al.

2006). The transducers measure the force applied between the tips of the forceps which is then displayed on a separate unit. Using the forceps a gradually increasing squeeze is placed on the paw of the animal. When the animal vocalises or withdraws from the apparatus the test is ended and the force applied is recorded. The application of a compression or squeeze force across the affected area is comparable to the Ritchie index and dolorimeter used in the clinic, but still does not focus on the knee joint due to the size of the apparatus and how wide the forceps would have to be opened to be applied to the knee joint.

In this chapter the development and validation of a pressure application measurement device (PAM) for use in both mice and rats is described (Strickland et al. 2005; Barton et al. 2007c). Adopting a similar principle to the clinical dolorimeter, a gradually increasing squeeze force is applied across the knee joint until the animal withdraws its limb from the apparatus. At this end-point the maximum force applied across the joint in grams is recorded to provide a quantitative objective measure of the sensitivity of the knee joint (see Section 2.2.2.).

The aim of the studies reported in this chapter was to evaluate the sensitivity of the readout in animals with a unilateral joint hypersensitivity and determine whether PAM could detect a reversal of this hypersensitivity when the animals were dosed with clinically effective analgesics. To validate the rat specific PAM device, a dose response study to prednisolone (Pyne et al. 2004), was conducted in parallel with single doses of morphine and celecoxib known to be effective as shown in previous studies (Caldwell et al. 2002; Schnitzer et al. 2005). A smaller study to validate the mouse specific PAM device was conducted investigating any analgesic effect of prednisolone (Gauldie et al. 2004). Throughout the studies results for PAM were compared with the already established WD results obtained from the incapacitance tester.

5.2 Methods

5.2.1 PAM apparatus

The first working PAM model was designed and built by Dr. H.M. Brash, a medical physicist at The University of Edinburgh, in collaboration with Prof. D.S. McQueen and Dr. A.W. Wilson (formerly of GlaxoSmithKline) who proposed the initial concept. The development and validation of the device was a project undertaken jointly by Dr. N.J. Barton and myself as part of independent Ph.D. studies (Strickland et al. 2005). At various stages through this project PAM was modified and fine-tuned to optimise its application based on feedback provided by Dr. N.J. Barton and myself. We developed two fully functioning PAM devices, one designed for use on rats and the other for use on mice (Barton et al. 2007c).

The PAM apparatus consists of a recording base unit containing a digital display and control buttons which is connected to a force transducer embedded in a moulded plastic thumb unit (Figure 5.1). The plastic unit is secured to the thumb using a Velcro fastened elasticated strap. Due to the difference in the size between a rat and a mouse knee joint, different force transducers were used in each species specific PAM device. In each case the force transducers have a circular contact area made of smooth, 1mm thick, rigid rubber. The rat instrument utilises a force transducer with a contact diameter of 8mm and surface area of 50.3mm² capable of measuring between 0 – 1500g (transducer - FSG-15N1A, Honeywell, Farnell, UK). The force transducer employed in the mouse PAM device has a contact diameter of 5mm, a surface area of 19.6mm², and has a measurable range of 0 – 500g (transducer - FSL-05N2C, Honeywell, Farnell, UK).



Figure 5.1 Photograph of the thumb unit of the pressure application measurement (PAM) device in use with a Male Wistar rat. The rat was lightly but securely held and the knee was subject to the gradually increasing squeeze force applied between the forefinger and force transducer until a point where the animal withdrew its limb from the apparatus.

5.2.2 Limb withdrawal threshold (LWT) assessment

The PAM device was used to assess the LWT as described in Section 2.2.2. At the point the animal withdraws from the apparatus the highest force applied is automatically displayed and manually recorded on a data sheet. The group mean LWT (gf) and the mean LWT ratio (\pm SEM) were calculated for each animal at each time point. Assistance was provided throughout by either Dr. N.J. Barton or Mrs. S.M. Bond.

5.2.3 Rat study design

An initial pilot study was performed in rats ($n=16$) to establish whether PAM was able to detect a localised hypersensitivity of the knee joint following an intra-articular injection of FCA (see Section 2.1.2). The aim of the study was to compare the LWTs of

FCA-injected knee joints with non-injected knee joints within the same animals, and with naïve animals. The study was also designed to compare PAM results with the already established weight distribution readout results. On each test day; the body weight, knee joint diameter, LWT and weight distribution were measured as described in section 2.2. Measurements followed a strict protocol to ensure the animals had breaks between measurements and did not become stressed by the procedure. Briefly, an animal was weighed and three consecutive weight distribution readings were taken and the animal was returned to the cage. Five minutes later, the animal had alternate measurements of the ipsilateral and contralateral limb assessed using PAM. This was repeated 3 times with a minute break in between each measurement. Finally the animal had its knee joint diameter measured. On day 0 baseline values were taken and animals were ranked, randomised and assigned to either the FCA (n=8) or the naïve (n=8) group, and received the appropriate treatment. Further measurements were then made 2-3 times a week up to day 28.

Following the pilot study, a larger drug study (n=56) was performed in our laboratory by Dr. N.J. Barton and Mrs. S.M. Bond to investigate whether PAM could be used to detect an analgesic effect expected from compounds with differing mechanisms of action. PAM was tested using the opioid morphine, the steroid prednisolone and the non-steroidal anti-inflammatory celecoxib, PAM was tested as to whether it could detect a reversal in the FCA-induced knee joint hypersensitivity as observed previously in the pilot study. In order to perform a thorough pharmacological investigation, whilst using the minimum number of animals, a dose response effect of prednisolone (1, 3 and 10mg.kg⁻¹) and single doses of morphine (3mg.kg⁻¹) and celecoxib (15mg.kg⁻¹) were examined with the drugs dosed chronically over a five day period.

As a consequence of the results observed in the pilot study, a pre-study behavioural assessment was introduced to habituate the animals to the PAM device. Baseline measurements were made on day 0 and subsequently animals were given either an intra-

articular injection of FCA (n=48) or naïve treatment (n=8). Measurements were made 2-3 times a week up to day 10. FCA-injected animals were ranked by how severe the joint hypersensitivity was on day 10, from most severe (low LWT ratio) to least severe (high LWT ratio). The animals were then assigned to one of 6 treatment groups ensuring that each group had an even spread of severity of FCA-induced hypersensitivity. The following drugs were then blindly dosed subcutaneously (s.c.) at a volume of 2ml.kg⁻¹ on days 14-18:

- Vehicle (ethanol 5%, Polyethylene Glycol [PEG] 45% and distilled water 50%)
- Prednisolone 1mg.kg⁻¹ (dosed once a day at 9am)
- Prednisolone 3mg.kg⁻¹ (dosed once a day at 9am)
- Prednisolone 10mg.kg⁻¹ (dosed once a day at 9am)
- Morphine 3mg.kg⁻¹ (dosed twice daily at 9am and 4pm)
- Celecoxib 15mg.kg⁻¹ (dosed twice a daily at 9am and 4pm)

One hour after the morning dose behavioural measurements were made with further behavioural assessments being carried out on days 21, 24 and 28.

5.2.4 Mouse study design

In addition to the two rat studies performed, a murine study (n=24) was carried out to validate the PAM device for mice. On each test day; the body weight, knee joint diameter, LWT and weight distribution were measured following the same protocol as the rat studies described above. Pre-injection measurements were made on day 0 and animals were assigned to either FCA (n=16) or naïve (n=8) action group. Following injection of FCA, animals had their behaviour, weight and knee joint diameter assessed 2-3 times a week up to day 10. The FCA-injected animals were then ranked by their day 10 LWT ratio and assigned to either the prednisolone (n=8) or vehicle (n=8) treatment group. On days 13 – 17 animals were dosed blindly once a day with either prednisolone 1mg.kg⁻¹ or vehicle (s.c.) at a volume of

3ml.kg⁻¹. Behavioural assessments took place one hour after dosing. After the completion of the dosing period, a final behavioural assessment was made on day 20.

5.2.5 Statistical analysis

Data were collected and collated in Microsoft Excel. The data were then analysed in GraphPad prism defined by the criteria stated in Section 2.5. Differences between two individual group mean values were analysed using a Wilcoxon t-test or an un-paired t-test depending on whether the data were paired or un-paired respectively. Results obtained from the behavioural studies were expressed as the group mean (\pm SEM) for both the WD and LWT ratios. The statistical test used to compare differences within the same group over time was a one-way ANOVA followed by a Dunn's post-hoc test as appropriate. The test used to compare differences between two or more different groups over time was the two-way ANOVA followed by Bonferroni's post-hoc test. In order to compare statistically the AUC values calculated from each dose of drug over the dosing period, a Kruskal-Wallis ANOVA was used followed by a Dunn's post-hoc to compare the different doses back to vehicle. In all cases $P \leq 0.05$ was considered statistically significant.

5.3 Results

In all the studies performed, the intra-articular injection of FCA had no adverse effects on the general health of the animals. Rats continued to gain body weight normally throughout the studies (Figure 5.2). There was no significant difference in the mean body weight within the FCA-injected group of rats compared to the naïve group at any time point ($P > 0.05$, two-way ANOVA).

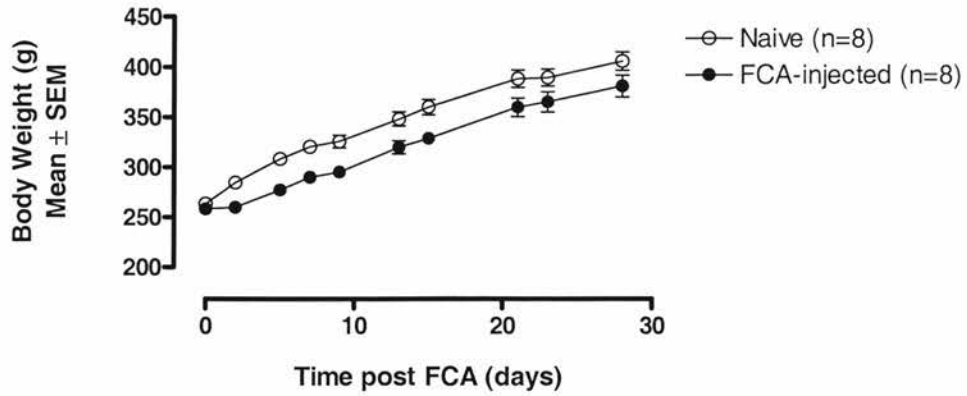


Figure 5.2 Figure illustrating the effect of an intra-articular injection on the body weights of rats in the pilot study. There was no significant differences in the group mean body weights between FCA-injected and naïve animals ($P>0.05$, two-way ANOVA). Following FCA injection (day 0) the animals did not gain as much weight over the following 24 hours as the naïve animals, but then gained weight normally thereafter. The group mean body weight of the FCA-injected animals was always slightly lower than that of naïve animals.

5.3.1 Pilot study in rats

5.3.1.1 Assessment of the limb withdrawal threshold

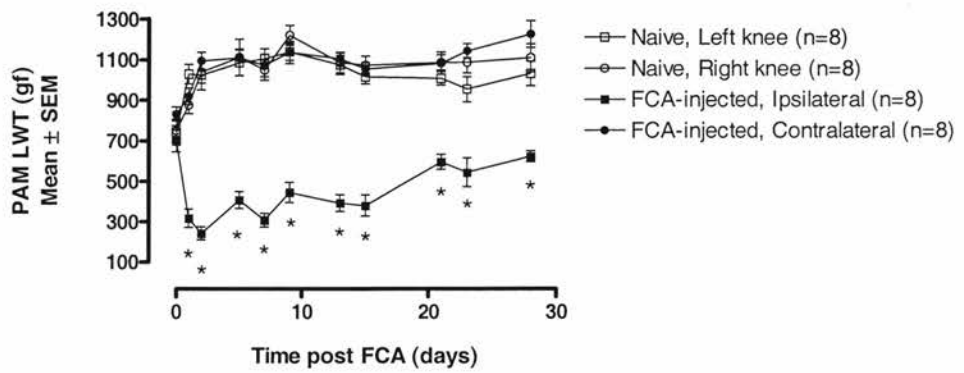
On day 0, baseline recordings revealed that the mean ipsilateral LWT was $720 \pm 31\text{gf}$ and the mean contralateral LWT was $791 \pm 34\text{gf}$, these were not significantly different from each other ($P>0.05$, Mann Whitney, $n=16$). Considering the baseline values with the animals in their assigned groups, there was no significant difference between the group mean LWTs for each individual limb ($P>0.05$, one-way ANOVA, $n=8$; Figure 5.3a), meaning there was no difference in the results obtained from the left and right knee, and no significant difference between the mean FCA LWT ratio and the mean naïve LWT ratio ($P>0.05$, Mann Whitney, $n=8$; Figure 5.4a).

In FCA-injected animals, the group mean ipsilateral LWT on day 1 decreased by 55% to $316 \pm 45\text{gf}$, a significant difference compared to both the previous day's group mean value ($P<0.05$, Wilcoxon) and also to the naïve group mean value at the same time point ($P<0.05$, Mann Whitney). Interestingly, the mean ipsilateral LWT of the naïve group increased significantly by 28% to $1028 \pm 48\text{gf}$ by day 1 compared to day 0 ($P<0.05$,

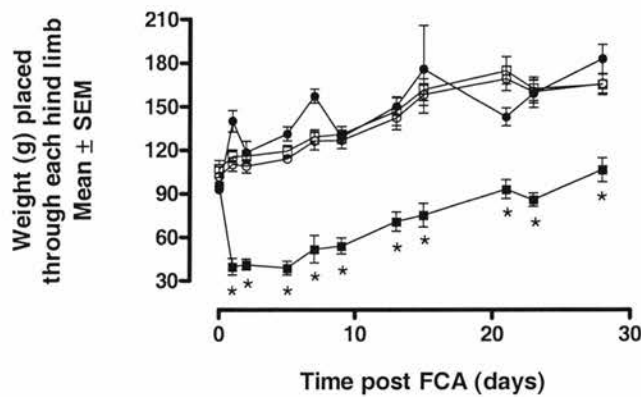
Wilcoxon). Similar increases in the LWT were observed in the contralateral limbs of both the FCA and naïve groups over the first 24 hours rising 10% and 15% respectively (see Figure 5.3a). The mean LWT ratio of the FCA-injected animals decreased a significant amount from the day 0 value of 0.87 ± 0.1 , to the day 1 value of 0.36 ± 0.07 ($P < 0.05$, Wilcoxon).

Throughout the 28 day test period, the mean ipsilateral LWT of the FCA-injected animals remained significantly lower than the naïve and contralateral mean LWTs ($P < 0.05$, two-way ANOVA; Figure 5.3a). Similarly the mean LWT ratio of the FCA-injected animals remained significantly lower than the mean LWT ratio of the naïve animals over the 28 day test period ($P < 0.05$, two-way ANOVA; Figure 5.4a).

(a)



(b)



(c)

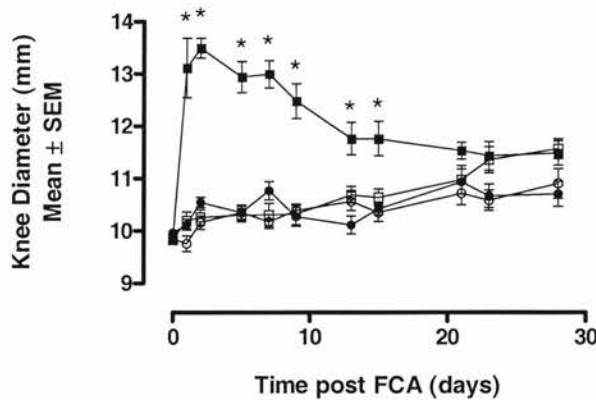


Figure 5.3 Figure showing the (a) PAM LWTs, (b) the weight carried by each hind limb over a three second period and (c) the knee joint diameter of naïve and FCA-injected rats over a 28 day pilot study. The inflammation of the FCA-injected limb was manifest as an increase in the knee joint diameter and was accompanied by a significant decrease in the LWTs and the amount of weight the animals placed through the limb. * represents statistical significance ($P < 0.05$) comparing each group at each time point using a two-way ANOVA followed by a Bonferroni post-hoc test.

5.3.1.2 Assessment of weight distribution

Prior to any treatment the mean weight carried by the ipsilateral and contralateral hind limbs was $101 \pm 3\text{g}$ and $97 \pm 4\text{g}$ respectively, giving a WD ratio of 0.96 ± 0.01 ($n=16$). At time 0 there was no significant difference between the group mean weight placed through any of the hind limbs ($P>0.05$, one-way ANOVA, $n=8$; Figure 5.3b) and no significant difference between the mean WD ratio of the FCA or naïve animals ($P>0.05$, Mann Whitney, $n=8$; Figure 5.4b).

The mean weight placed through the ipsilateral and contralateral hind limbs of FCA-injected animals on day 1 was $40 \pm 7\text{g}$ and $140 \pm 7\text{g}$ respectively. The ipsilateral value was significantly lower than the previous days group mean value ($P<0.05$, Wilcoxon) and also to the naïve ipsilateral group mean value at the same time point ($P<0.05$, Mann Whitney). Subsequently, the mean WD ratio of the FCA group fell by 63% to 0.36 ± 0.04 . This was significantly lower than in the naïve group where animals placed an average of $116 \pm 4\text{g}$ and $110 \pm 5\text{g}$ through the ipsilateral and contralateral limbs respectively, a WD ratio of 1.07 ± 0.03 ($P<0.05$, Mann Whitney, $n=8$).

The mean weight placed through the FCA-injected limb remained significantly lower than the naïve and both contralateral hind limb weights throughout the 28 day period ($P<0.05$, two-way ANOVA; Figure 5.3b). Similarly the WD ratio of the FCA group remained significantly lower than the naïve group on each test over the test period ($P<0.05$, two-way ANOVA; Figure 5.4b).

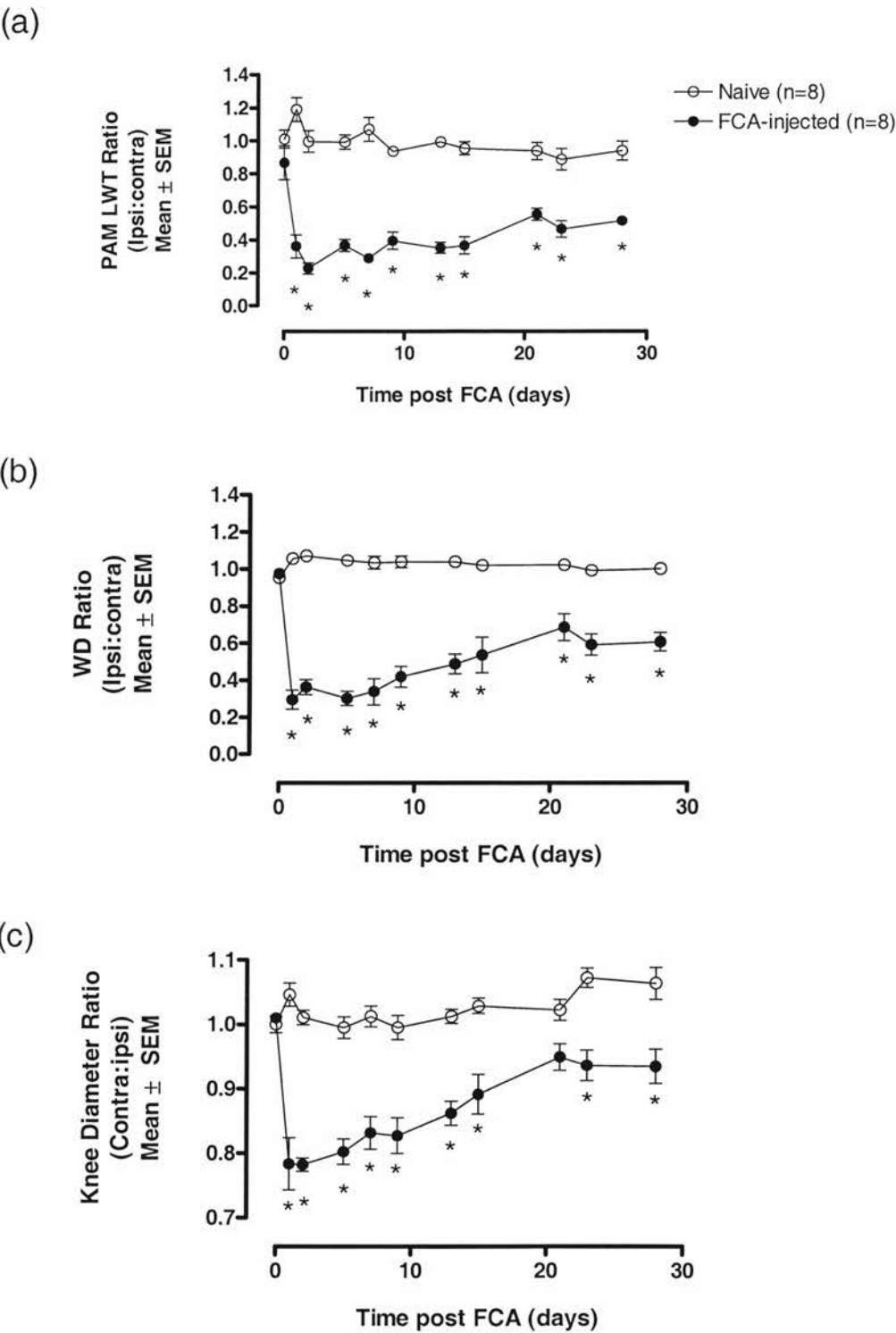


Figure 5.4 Figure showing the (a) PAM LWT ratios, (b) the weight distribution ratios and (c) the knee joint diameter ratios of rats over a 28 day pilot study. The PAM LWT ratio of both FCA and naïve animals had a similar 28 day profile to that of the weight distribution ratio. * represents statistical significance ($P<0.05$) comparing each group at each time point using a two-way ANOVA followed by a Bonferroni post-hoc test.

5.3.1.3 Assessment of inflammation induced by FCA

Baseline recordings of the ipsilateral and contralateral knee joint diameter taken on day 0 were $9.86 \pm 0.05\text{mm}$ and $9.91 \pm 0.07\text{mm}$ respectively. There was no significant difference between these values ($P>0.05$, Mann Whitney, $n=16$). The mean knee joint diameter ratios on day 0 were 1.00 ± 0.01 in the naïve group and 1.01 ± 0.01 in the FCA group again with no significant difference between the groups ($P>0.05$, Mann Whitney, $n=8$).

On day 1 the mean diameter of the FCA-injected knee was $13.11 \pm 0.56\text{mm}$, an increase of 25% from the previous day and a significantly higher value than the naïve ipsilateral value of $10.20 \pm 0.16\text{mm}$ ($P>0.05$, Mann Whitney, $n=8$; Figure 5.3c). The peak mean joint diameter of the FCA-injected knee was $13.50 \pm 0.19\text{mm}$ and observed on day 2. Following this reading the knee joint diameter gradually returned towards naïve and contralateral levels.

The swelling in the FCA-injected knee remained significantly greater than the naïve animals on days 1-15 ($P<0.05$, two-way ANOVA; see Figure 5.3c). The knee joint diameter ratio of the FCA group remained significantly higher than that of the naïve animals on all days in the 28 day test period with the exception of day 21 ($P<0.05$, two-way ANOVA; Figure 5.4c).

5.3.1.4 Correlation of rat PAM LWT ratio and WD ratio

In order to compare the LWT and WD readouts directly, the ratios obtained on the 11 experimental days over the 28 day study period were plotted against each other on an XY plot with a linear regression line (see Figure 5.5). The ratios were used because in both readouts the contralateral limb acts as an internal control, having previously established that it didn't change. A strong positive correlation between the ratios obtained from each readout was observed using Spearman's Linear correlation (Spearman $r=0.76$, $P<0.0001$).

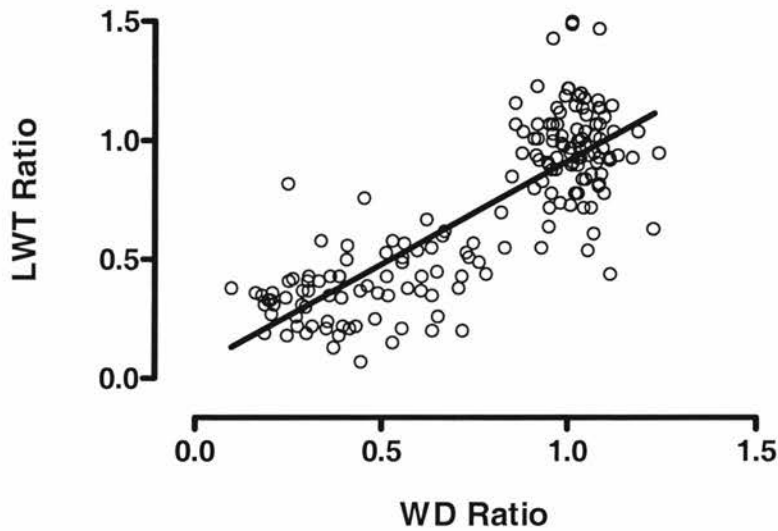


Figure 5.5 Plot showing the correlation between the PAM LWT ratios and the WD ratios obtained from the same animals during the rat pilot study. There was a strong positive correlation between the two readouts, which was highly significant (Spearman $r=0.76$, $P<0.0001$, $n=176$, 16 measurements per day for 11 days)

5.3.2 Drug studies in rats

5.3.2.1 Assessment of the limb withdrawal threshold

The study discussed in section 5.3.2 was performed by Dr. N. Barton and Mrs. S. Bond, although the analysis of results was performed independently. Prior to FCA-injection the mean ipsilateral LWT was $1066 \pm 24\text{gf}$ and the mean contralateral LWT was $1037 \pm 21\text{gf}$, which were not significantly different from each other ($P>0.05$, Wilcoxon, $n=55$). There was no significant difference between the mean LWT ratios in any of the dosing groups at this time point ($P>0.05$, one-way ANOVA, $n=8$).

FCA resulted in a joint hypersensitivity which was apparent by day 2 and was still present on day 10. On days 2, 4, 7 and 10 the mean FCA-injected LWTs were significantly lower than the corresponding contralateral LWTs in the same animals and the LWTs of naïve animals ($P<0.05$, two-way ANOVA, $n=47$; Figure 5.6a). The mean group LWT ratios of FCA-injected animals were significantly lower than the naïve LWT ratios at each time point through days 2-10 ($P<0.05$, two-way ANOVA, $n=47$; Figure 5.6d).

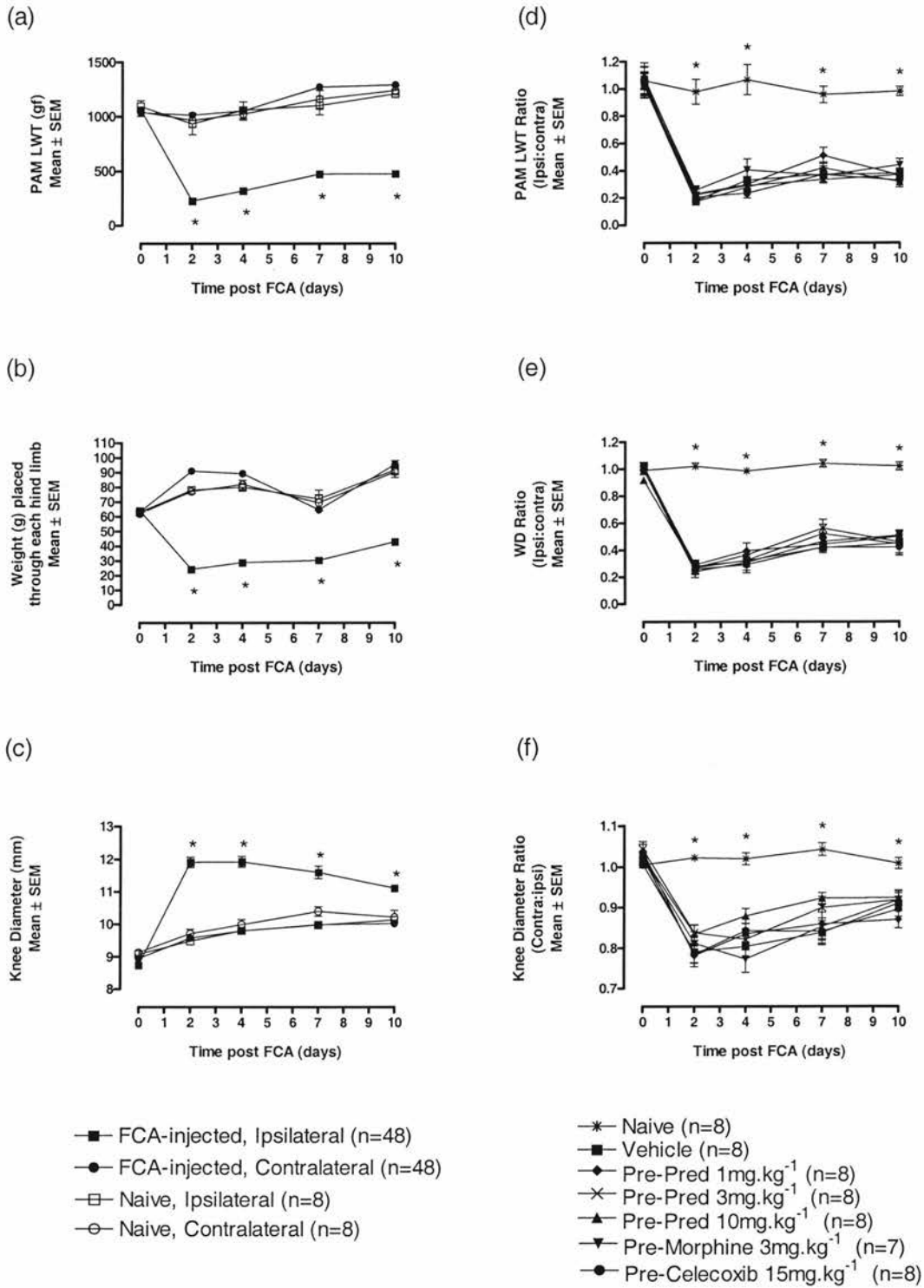


Figure 5.6 Summary of the rat mean values of the absolute (a) PAM LWTs, (b) weight placed through each hind limb and (c) knee joint diameters for each limb following naïve or FCA treatment from day 0 up to day 10, when drug treatment commenced. The mean group ratios of (d) PAM LWTs, (e) weight distribution and (f) knee joint diameter of the individual dosing groups from day 0 to day 10. * represents statistical significance ($P<0.05$) comparing the groups at each time point using a two-way ANOVA followed by a Bonferroni post-hoc test.

Drugs were administered daily for a five day period starting on day 14 (Figure 5.7a & 5.8a). AUC values for the LWT ratios over the 5 day dosing period were calculated for each individual animal, using the day 10 reading as the baseline, to observe if there was any reversal of hypersensitivity due to the drugs being administered compared to vehicle. Results showed that PAM was able to detect the expected significant analgesic effect of morphine 3mg.kg^{-1} , celecoxib 15mg.kg^{-1} and the top dose of prednisolone during the dosing period ($P<0.05$, one-way ANOVA, $n=8$; Figure 5.7c & 5.8c).

Following the cessation of the dosing period on day 18, further assessments were made on day 21, 24 and 28. The analgesic effects observed during the dosing period were absent on days 21, 24 and 28, and the LWT ratios of the drug groups returned to not being significantly different to the vehicle group ($P>0.05$, two-way ANOVA, $n=8$).

5.3.2.2 Assessment of weight distribution

There was no significant difference ($P>0.05$, Wilcoxon, $n=55$) between the weight placed through the limbs to be injected with FCA ($64 \pm 1\text{gf}$) compared to the contralateral limbs ($63 \pm 1\text{gf}$) prior to FCA-injection. There was also no significant difference between the mean WD ratios of each of the dosing groups at this time point ($P>0.05$, one-way ANOVA, $n=8$).

The injection of FCA caused a joint hypersensitivity that was present at days 2 - 10. Animals placed significantly less weight through the FCA-injected limb than the corresponding contralateral limb in the same animals and both limbs of naïve animals ($P<0.05$, two-way ANOVA, $n=47$; Figure 5.6b). The mean group WD ratios of FCA-injected animals were significantly lower than the naïve WD ratios at each time point ($P<0.05$, two-way ANOVA, $n=47$; Figure 5.6e).

A five day dosing period began on day 14 where drugs were administered daily followed by the behavioural assessments one hour later (Figure 5.7b & 5.8b). The WD ratios

for each individual animal over the 5 day dosing period were transformed into AUC values, using the day 10 reading as the baseline. Any analgesic effect of the drugs was investigated by comparing the AUC values for each dosing group to the AUC values from the vehicle group. Results showed that the incapacitance tester detected the analgesic effect of morphine 3mg.kg^{-1} , celecoxib 15mg.kg^{-1} and the top dose of prednisolone (10mg.kg^{-1}) during the dosing period ($P<0.05$, one-way ANOVA, $n=8$; Figure 5.7d & 5.8d).

Following the cessation of the dosing period, the WD ratios of the drug groups on days 21, 24 and 28 reverted to those of the vehicle group. The reduction of hypersensitivity observed as a result of administering the drugs was absent and dosing groups were no longer significantly different to the vehicle group ($P>0.05$, two-way ANOVA, $n=8$).

5.3.2.3 Assessment of inflammation induced by FCA

There was no significant difference ($P>0.05$, Wilcoxon, $n=55$) between the knee joint diameter of the ipsilateral limbs ($8.81 \pm 0.13\text{mm}$) compared to the contralateral limbs ($8.97 \pm 0.09\text{mm}$) prior to FCA-injection. There was also no significant difference between the mean knee joint diameter ratios of each of the dosing groups on day 0 ($P>0.05$, one-way ANOVA, $n=8$).

FCA resulted in a unilateral inflammation that caused the ipsilateral knee becoming significantly greater in diameter than contralateral and naïve knee joint diameters on days 2, 4, 7 and 10 post-FCA ($P<0.05$, two-way ANOVA, $n=47$; Figure 5.6c). The mean group knee joint diameter ratios of FCA-injected animals were significantly lower than the naïve knee joint diameter ratios at each time point through days 2-10 ($P<0.05$, two-way ANOVA, $n=47$; Figure 5.6f).

Administration of morphine 3mg.kg^{-1} , celecoxib 15mg.kg^{-1} , or prednisolone at 3 or 10mg.kg^{-1} did not significantly affect the swelling observed in the ipsilateral limbs of FCA-injected animals compared to vehicle ($P>0.05$, one-way ANOVA, $n=8$; Figure 5.9).

Prednisolone at 1mg.kg^{-1} did however reduce the amount of swelling in the ipsilateral limbs compared to vehicle, seen as a significant increase in the AUC group value compared to vehicle ($P<0.05$, one-way ANOVA, $n=8$; Figure 5.9)

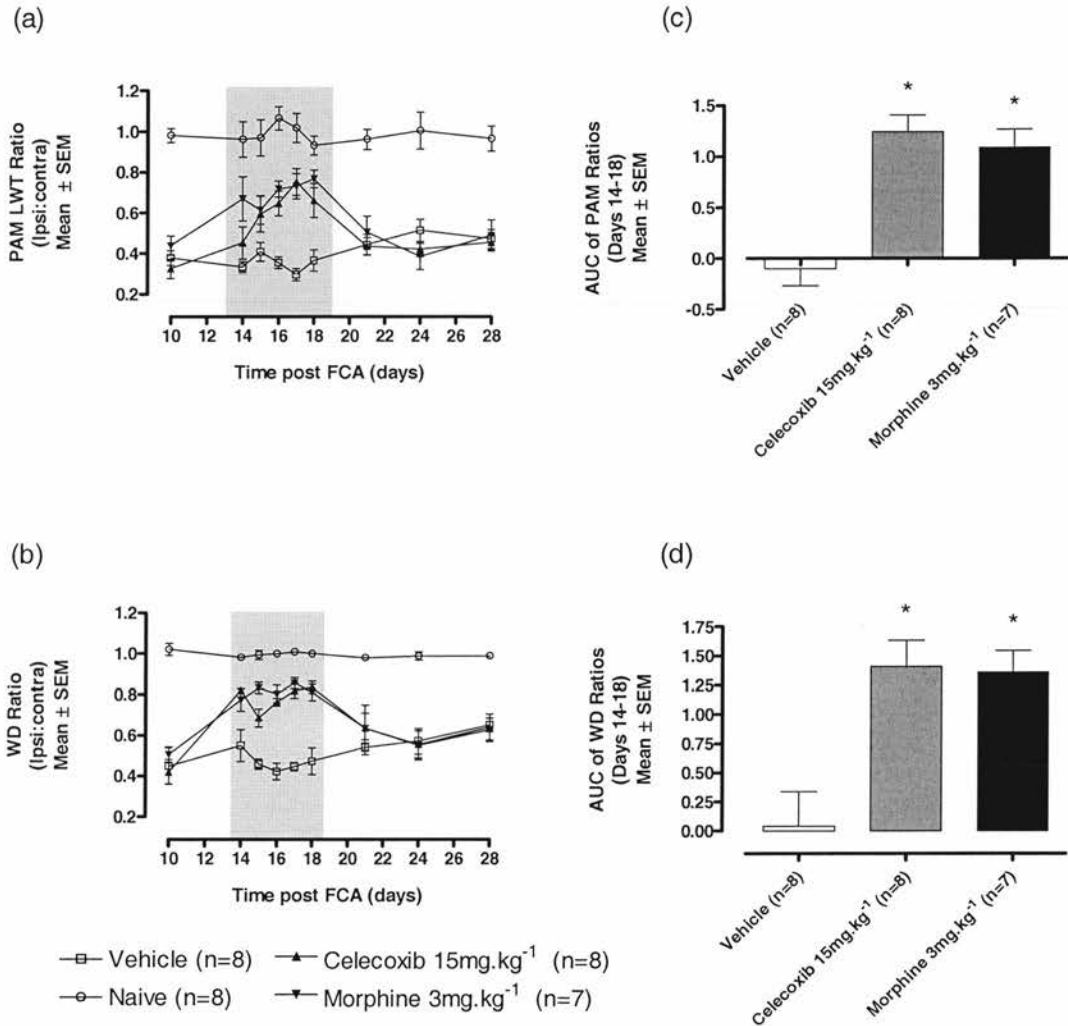


Figure 5.7 Figure showing the mean (a) PAM LWT ratios and (b) WD ratios of the morphine-, celecoxib- and vehicle-treated animals following injection of FCA from day 10 through to day 28. The dosing period (day 14-18) is indicated by the shaded area. The corresponding PAM and WD ratio AUC graphs (c & d) during the dosing period were analysed to compare the celecoxib (15mg.ml^{-1}) and morphine (3mg.ml^{-1}) treated groups to the vehicle-treated group. * represents statistical significance ($P<0.05$) comparing the dosing groups to vehicle using a one-way ANOVA followed by Dunn's post-hoc test.

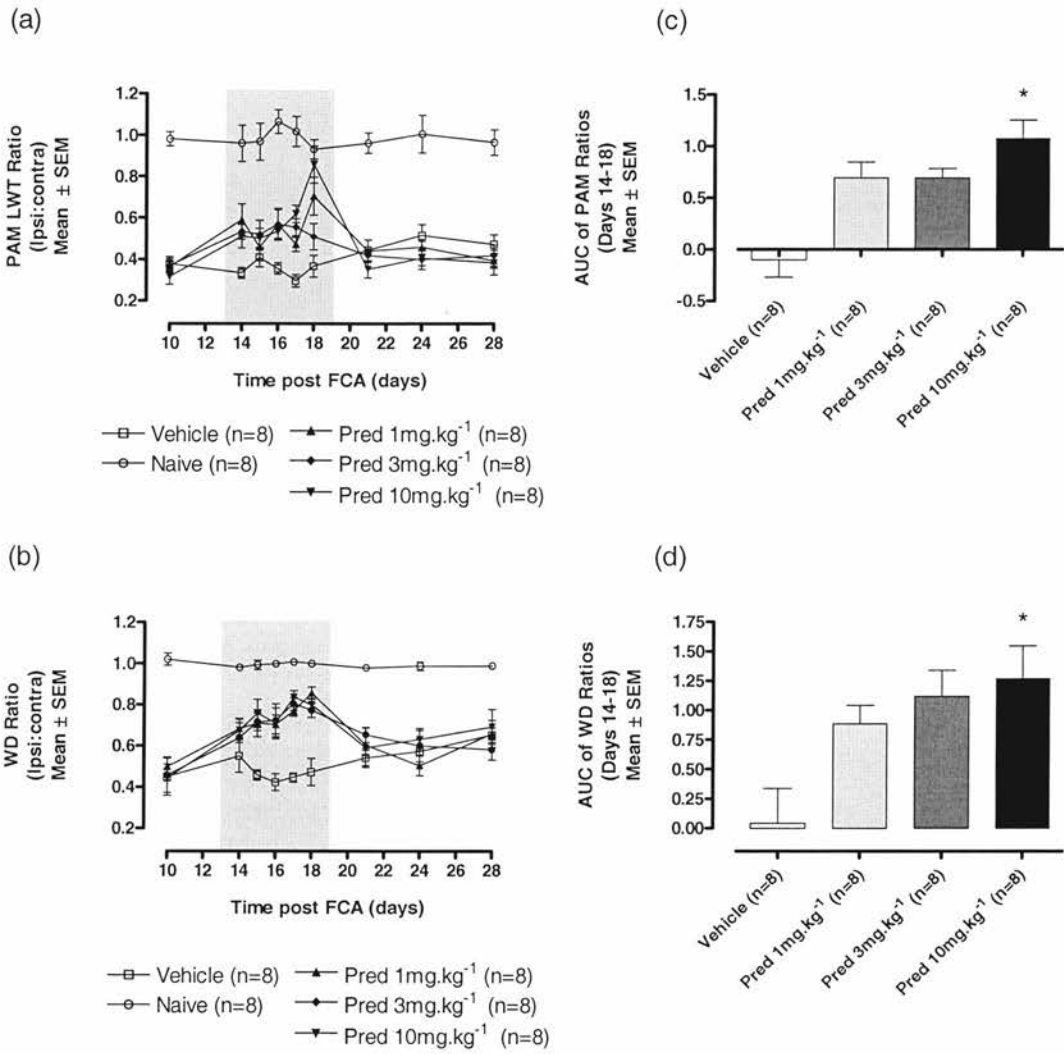


Figure 5.8 Figure showing the mean (a) PAM LWT ratios and (b) WD ratios of the prednisolone- and vehicle-treated animals following injection of FCA from day 10 through to day 28. The dosing period (day 14-18) is indicated by the shaded area. The corresponding PAM and WD ratio AUC graphs (c & d) during the dosing period were analysed to compare the prednisolone 1-10mg.kg⁻¹ groups to the vehicle-treated group. * represents statistical significance ($P<0.05$) comparing the dosing groups to vehicle using a one-way ANOVA followed by a Dunn's post-hoc test.

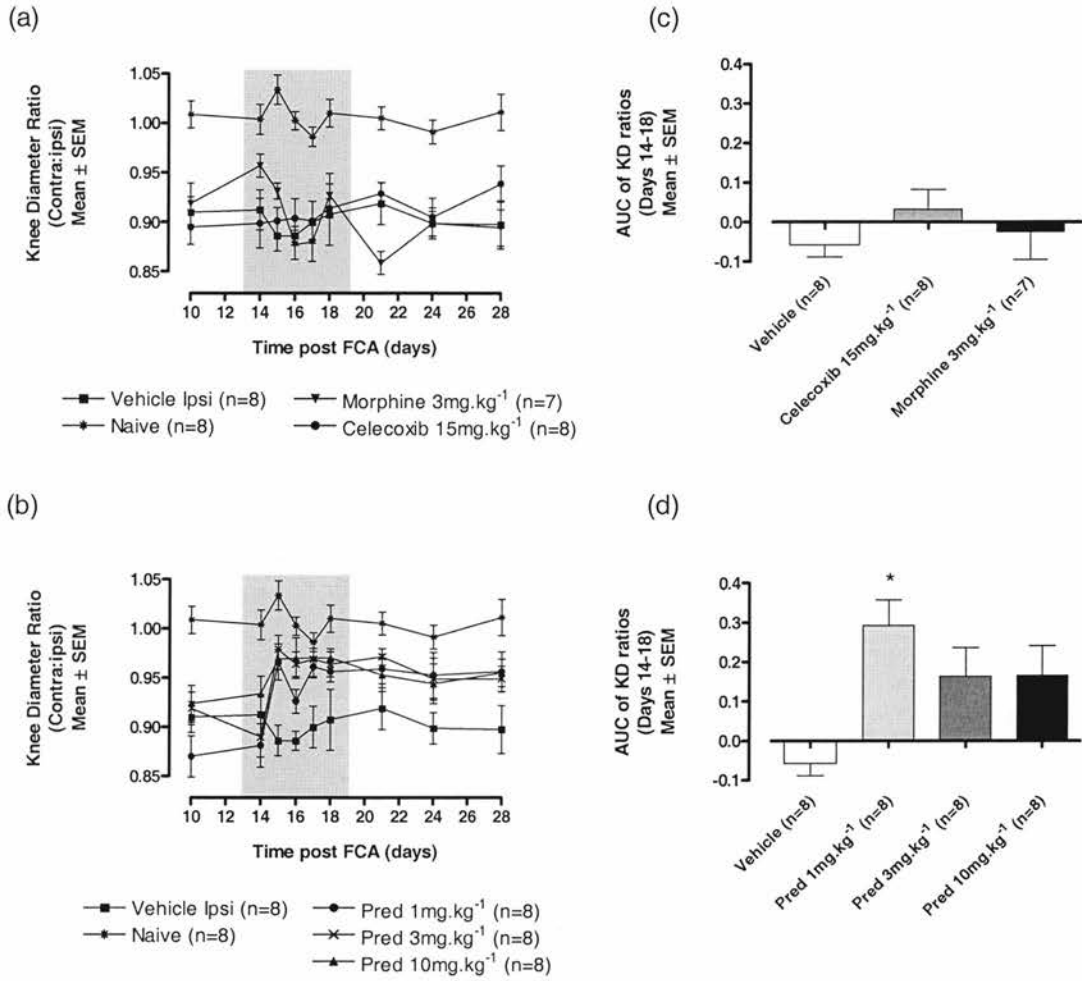


Figure 5.9 Plots showing the mean knee joint diameter ratios observed at days 10–28 post-FCA and the accompanying AUC ratio graphs from the dosing period of the rat drug study for (a & c) celecoxib and morphine and (b & d) prednisolone. The dosing period (day 14–18) is indicated by the shaded area. * represents statistical significance ($P < 0.05$) using a one-way ANOVA followed by Dunn's post-hoc test to compare the vehicle group to each dosing group.

5.3.3 Pilot study in mice

5.3.3.1 Assessment of the limb withdrawal threshold

On day 0 the ipsilateral and contralateral mean LWTs of mice were 414 ± 10 gf and 381 ± 14 gf respectively and were not significantly different ($P > 0.05$, Wilcoxon, $n = 24$). When analysing the mean LWT ratios of the assigned groups; FCA + prednisolone 1mg.kg^{-1} , FCA + vehicle, and naïve there was no significant difference between the group mean values of 1.14 ± 0.09 , 1.21 ± 0.1 and 1.15 ± 0.1 respectively ($P > 0.05$, one-way ANOVA, $n = 8$).

By day 1 post-FCA the mean ipsilateral LWT of FCA-injected animals decreased by 60% to $162 \pm 9\text{gf}$ compared to the day 0 values ($P < 0.05$, Wilcoxon, $n=16$, Figure 5.10a) and was significantly lower than naïve at the same time point ($P < 0.05$, Mann Whitney, $n=8$). The PAM LWT ratio of the FCA injected animals was also significantly reduced over the 1 day period from 1.17 ± 0.27 to 0.63 ± 0.04 ($P < 0.05$, Wilcoxon, $n=8$, Figure 5.10d). Surprisingly there was a significant decrease in the LWTs of contralateral limbs from day 0 to day 1 with the group mean LWT decreasing by 22% to $297 \pm 12\text{gf}$ ($P < 0.05$, Wilcoxon, $n=8$, Figure 5.10a). The group mean LWT ratio of the naïve animals did not change significantly following FCA. The mean LWT ratios of FCA-injected animals remained significantly lower than naïve animals on days 3 and 10 ($P < 0.05$, two-way ANOVA, $n=24$; Figure 5.10d).

Prednisolone caused a reversal of hypersensitivity in FCA-injected mice that was significantly different to vehicle on days 16 and 17 ($P < 0.05$, two-way ANOVA, $n=24$; Figure 5.11a). The mean AUC values calculated for prednisolone 1mg.kg^{-1} and vehicle over the 5 day dosing period were significantly different ($P < 0.05$, Mann Whitney, $n=8$; Figure 5.11d). Following the cessation of dosing the LWT ratio of the prednisolone group on day 20 reverted back to being no longer significantly different to the vehicle group ($P > 0.05$, Mann Whitney, $n=8$).

5.3.3.2 Assessment of weight distribution

The mean weight placed through the ipsilateral and contralateral limbs was $9.9 \pm 1\text{g}$ and $9.8 \pm 1\text{g}$ respectively, which were not significantly different from each other ($P > 0.05$, Wilcoxon, $n=24$). On day 0 the group mean WD ratios of the prednisolone, vehicle and naïve groups were 1 ± 0.03 , 1 ± 0.03 and 1 ± 0.02 respectively and were not significantly different ($P > 0.05$, one-way ANOVA, $n=8$).

Injection of FCA caused the mean weight placed through the ipsilateral limb of FCA-injected animals to fall significantly to $7.22 \pm 0.49\text{g}$. This was significantly lower than

the day 0 values ($P < 0.05$, Wilcoxon, $n=16$, Figure 5.10b) and naïve at the same time point ($P < 0.05$, Mann Whitney, $n=8$). The WD ratio of the FCA injected animals was also significantly reduced over the 24 hours from 1.01 ± 0.20 to 0.64 ± 0.06 ($P < 0.05$, Wilcoxon, $n=8$, Figure 5.10b). The mean WD ratio of FCA-injected animals remained significantly lower than naïve animals on days 1, 3 and 10 post-FCA ($P < 0.05$, two-way ANOVA, $n=24$; Figure 5.10e).

During the dosing period prednisolone reversed hypersensitivity in FCA-injected mice, such that the LWT ratio was significantly higher to vehicle on day 17 ($P < 0.05$, two-way ANOVA, $n=24$; Figure 5.11a). The mean AUC values calculated for prednisolone 1mg.kg^{-1} and vehicle over the 5 day dosing period were significantly different ($P < 0.05$, Mann Whitney, $n=8$; Figure 5.11e). Following the cessation of dosing the WD ratio of the prednisolone group on day 20 was no longer significantly different to that of the vehicle group ($P > 0.05$, Mann Whitney, $n=8$).

5.3.3.3 Assessment of inflammation induced by FCA

The mean ipsilateral and contralateral knee joint diameters taken prior to FCA-injection were $4.02 \pm 0.02\text{mm}$ and $4.01 \pm 0.02\text{mm}$ and were not significantly different from each other ($P > 0.05$, Wilcoxon, $n=24$). Injection of FCA ($n=16$) caused the mean ipsilateral knee joints to swell significantly ($P < 0.05$, one-way ANOVA; Figure 5.10c) to $4.82 \pm 0.10\text{mm}$, compared to both the naïve group ($4.13 \pm 0.06\text{mm}$, $n=8$) and all the contralateral limbs ($4.05 \pm 0.02\text{mm}$, $n=24$) at the same time point. The group mean knee joint diameter ratios of the FCA-injected animals ($n=16$) remained significantly lower than naïve animals ($n=8$) up to day 10 ($P < 0.05$, two-way ANOVA; Figure 5.10f).

Prednisolone had no significant effect on the knee joint diameter ratio on any day during the dosing period or on the mean AUC values calculated during this time period ($P > 0.05$, two-way ANOVA and Mann Whitney; Figure 5.11c & 5.11f).

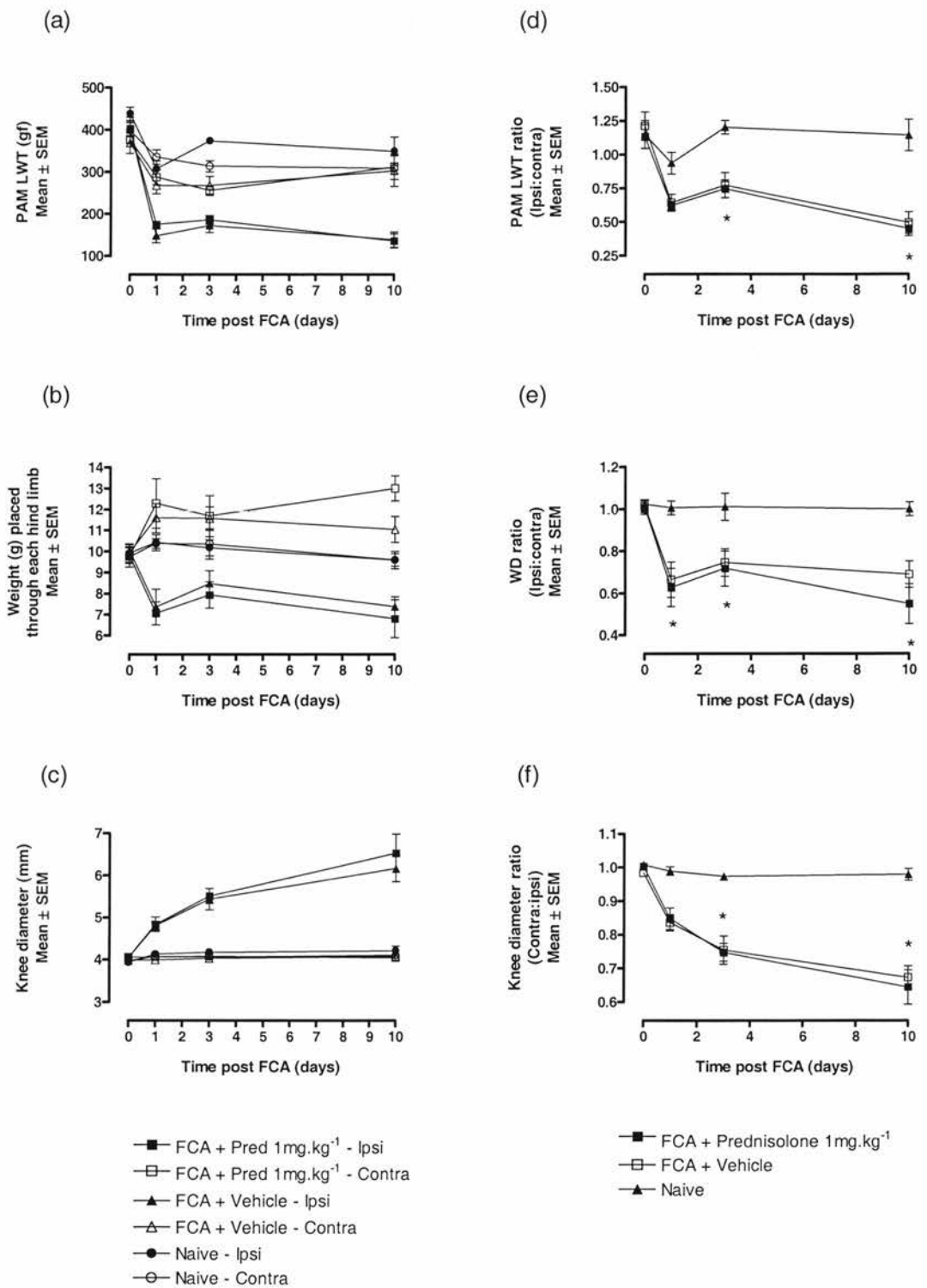


Figure 5.10 Figure showing the mean values of the absolute (a) PAM LWTs, (b) weight placed through each hind limb and (c) knee joint diameters in mice for each limb following naïve or FCA treatment from day 0 up to day 10 prior to drug treatment. The mean group ratios of (d) PAM LWTs, (e) weight distribution and (f) knee joint diameter of the individual dosing groups from day 0 to day 10. * represents statistical significance ($P<0.05$) comparing the groups at each time point using a two-way ANOVA followed by a Bonferroni post-hoc test.

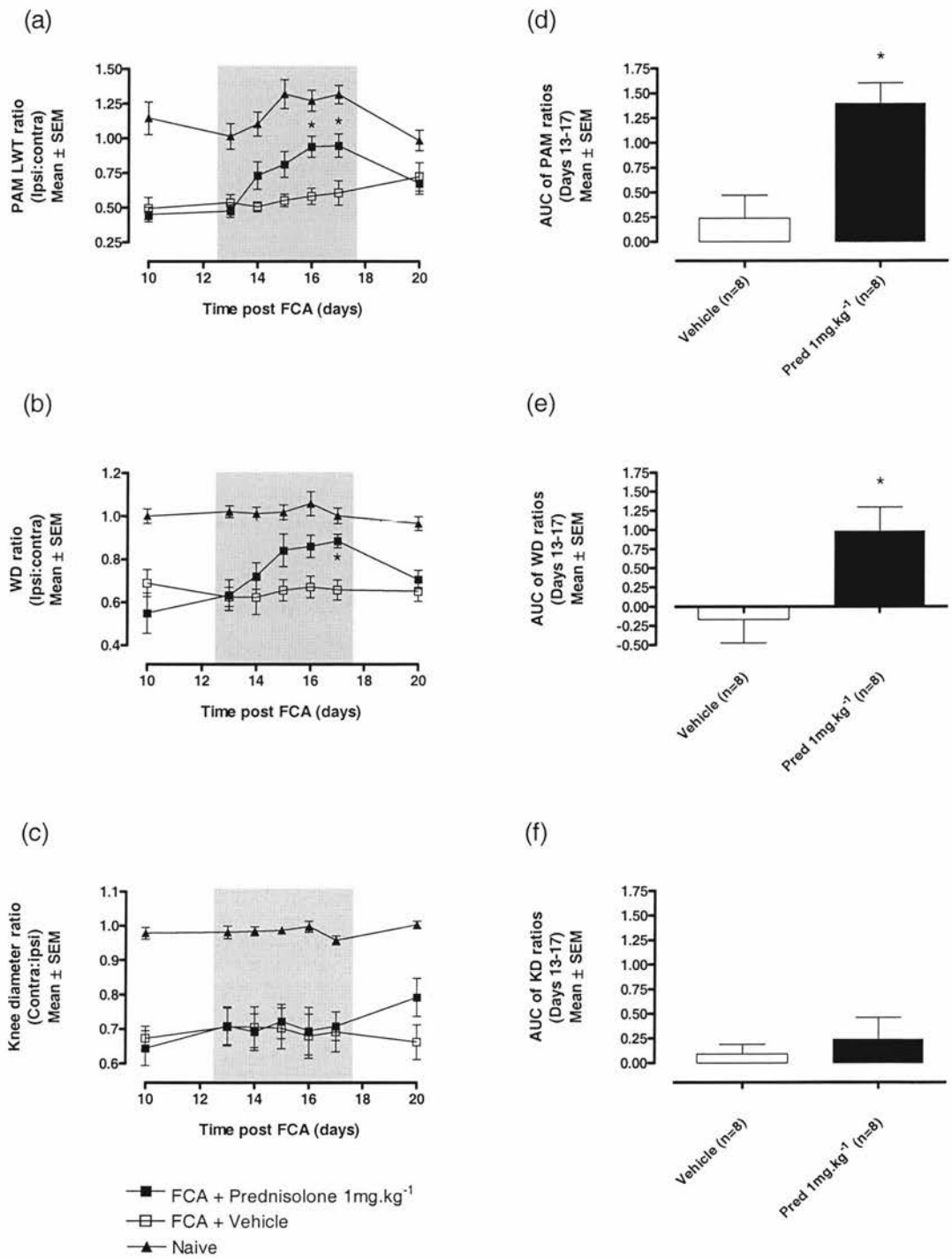


Figure 5.11 Summary of results performed using mice showing the mean (a) PAM LWT ratios, (b) WD ratios and (c) knee diameter ratios of the prednisolone (1mg.kg⁻¹) and vehicle-treated animals following injection of FCA from day 10 through to day 20. The dosing period (day 13-17) is indicated by the shaded area. * represents statistical significance ($P < 0.05$) comparing the prednisolone group to vehicle using a two-way ANOVA followed by a Bonferroni post-hoc test. The corresponding PAM, WD and knee joint diameter ratio AUC graphs (d, e and f) during the dosing period were analysed to compare the drug-treated groups to the vehicle-treated group. * represents statistical significance ($P < 0.05$) comparing the prednisolone group to vehicle using a Mann Whitney test.

5.3.3.4 Correlation of mouse PAM LWT ratio and WD ratio

In order to compare the two different readouts investigated in the mouse pilot study a correlation of the LWT ratio results and WD ratio results was calculated. The ratios measured over the 10 experimental days of the 20 day study period were plotted against each other (see Figure 5.12). A strong positive correlation between the readouts was observed using Spearman's Linear correlation (Spearman $r=0.66$, $P<0.0001$).

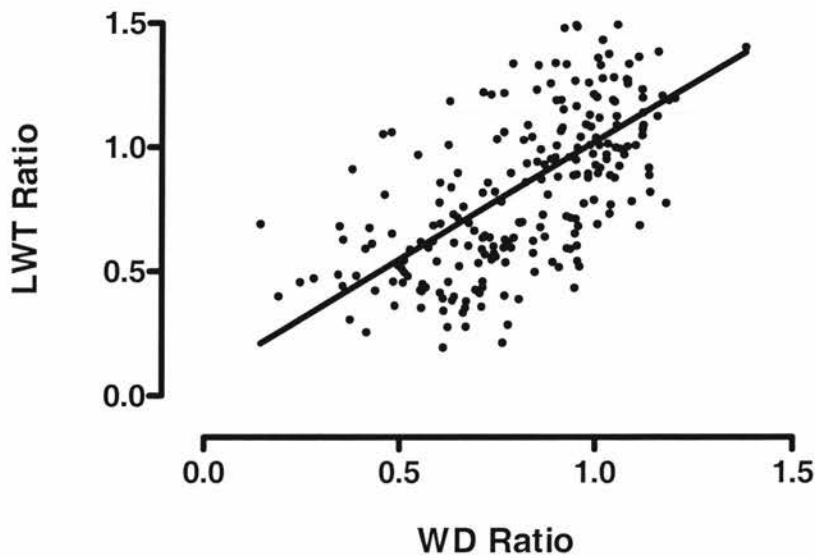


Figure 5.12 Plot of the correlation between the PAM LWT ratios (FCA-injected:contralateral) and the WD ratios (FCA-injected:contralateral) obtained from matched animals during the mouse pilot study. There was a positive correlation between the two readouts, which was highly significant (Spearman $r=0.66$, $P<0.0001$, $n=240$, 24 measurements per day for 10 days)

5.4 Discussion

This chapter describes how the PAM device has been developed and validated as a novel behavioural tool for assessing knee sensitivity in two rodent models of chronic inflammatory joint pain (Strickland et al. 2005; Barton et al. 2007c). Results obtained in these studies show that PAM provides a reliable, quantitative measurement of knee joint hypersensitivity in both mice and rats following an induction of joint inflammation as a result of an intra-articular injection of FCA. Furthermore, the PAM apparatus can be used to detect the analgesic action of prednisolone in mice and rats, and of morphine and celecoxib in rats, manifested as a reduction in joint hypersensitivity compared to vehicle. A strong correlation was observed between the LWT ratios calculated from PAM and the WD ratios calculated from the incapacitance tester in both species, indicating that PAM will be a valuable screening tool for testing novel analgesics in pre-clinical animal models of joint pain.

The practical use of PAM was a very simple procedure for both the user and the animal being tested. A straightforward holding or scruffing technique is all that was required to prepare the animal for a LWT measurement to be taken. On average the time taken from picking the animal out of its cage to placing it back, having taken both an ipsilateral and contralateral reading, is estimated as being approximately thirty seconds. This meant that screening a large number of animals in one session was a feasible prospect. Handling and the initial testing of the animals, is arguably a stressful experience for the animals and therefore habituation tests were introduced to try and familiarise them to the experience. Stress is a response to a potentially threatening situation and can lead to analgesia (Takahashi 1991) as a result of the activation and subsequent dysregulation of the hypothalamic-pituitary-adrenocortical axis. Radiotelemetry devices may be used in future studies to monitor blood pressure in the conscious animals, giving an indication of how stressful the handling and testing process is (Kramer and Kinter 2003). Unfortunately the cost of such devices, and the

time taken to surgically implant them, makes this method of monitoring the animals unfeasible in a laboratory of our size. The major disadvantage of the PAM device was that two people are required at all times, one person to hold the animal and a second person to operate PAM. For the majority of behavioural readouts only one person is normally required to take the measurements (Randall and Selitto 1957; Chaplan et al. 1994; Clayton et al. 1997). The same personnel were used for holding the animal and operating the PAM device throughout the study, with the roles maintained throughout. This practice ensured any operator differences were avoided.

PAM was modified throughout its development with the size of the rubber contact pad in particular receiving fine-tuning. Pressure exerted onto the skin above the knee joint may activate nociceptive afferents in several tissues, depending on the shape and surface area of the contact. A small contact area may have resulted in the exclusive activation of cutaneous nociceptive fibres innervating the skin, in much the same way as von Frey hairs (Chaplan et al. 1994; Khalsa et al. 1997), whereas adopting a larger contact area resulted in the preferential activation of deep tissue afferents within the knee joint, because the force applied is spread out over the skin (Treede et al. 2002). Experiments using a topical local anaesthetic have shown that the contribution of cutaneous afferents to pain evoked by a flat blunt pressure is minimal (Kosek et al. 1995).

Pressure is defined as the force per unit area applied on a surface, perpendicular to that surface. It was decided not to present the data obtained from PAM in units of pressure as it cannot be assumed that the entire surface of the rubber contact is always evenly apposed on the side of the joint, and furthermore as a squeeze pressure is applied, the joint will deform and this will occur differently in inflamed and non-inflamed joints. Therefore the decision was taken to express results obtained from PAM in terms of the force in grams applied (gf) and it was therefore essential to present the results together with information about the size of the rubber contact surface used on the joint. For example, if we applied a

force of 300g, a contact diameter of 1mm would result in the force being applied over a pointed area of $7.86 \times 10^{-3} \text{ mm}^2$, and would result in a pressure spot of $3.8 \times 10^4 \text{ gf.mm}^{-2}$. If the same force were then to be applied using a circular contact with a diameter of 8mm, the force will be applied over a flattened area of 50.3 mm^2 , and would result in a much lower pressure of 5.97 gf.mm^{-2} .

In the studies described here the rat PAM device used a circular rubber contact pad with a diameter of 8mm and the mouse PAM device used a circular rubber contact pad with a diameter of 5mm. If it were assumed that the knee did not deform and that the force applied was distributed evenly over the rigid rubber contact, then the pressure applied at the point of limb withdrawal was approximately 20 gf.mm^{-2} in both species. Similar values were reported using the Randall and Selitto analgesy-meter where a circular contact plinth of diameter 1.4mm was used to obtain naïve rat LWT results of 60g giving a pressure applied of approximately 40 gf.mm^{-2} (Anseloni et al. 2003). Furthermore values obtained using calibrated forceps in both behavioural and electrophysiological studies of the rat report a noxious pressure of the paw being in the region of 50 gf.mm^{-2} (Neugebauer and Li 2002; Luis-Delgado et al. 2006). Although differences do occur in the approximate values, this is probably due to differences in the strain and weight of the animals and more importantly the gross anatomical differences of the paw and knee.

To date the only other behavioural assessment described capable of directly measuring knee joint sensitivity involves the use of calibrated forceps equipped with strain gauges (Yu et al. 2002; Han et al. 2005; Skyba et al. 2005). The authors describe the forceps for use on rats only, which may mean that they are not suitable for use with mice. PAM however, is capable of being used in both species. The contact area of the forceps was blunt and had a surface area of 16 mm^2 (4mm long x 4mm wide). This surface contact area was considerably smaller than that of 50.3 mm^2 that was utilised in the rat PAM device. Moreover the contact surface of the forceps was exposed metal, whereas the PAM contact area was a

rigid rubber disc. The differences in the size and finish of the contact area may lead to the devices activating different proportions of nociceptive afferents in the skin and deep tissues as described above. The large padded contact area used in PAM provides a measurement more representative of a 'compression' of the whole knee joint rather than the small contact area of the forceps which may in fact be investigating a 'pinch' of a small portion of the knee joint (Skyba et al. 2005). PAM also has the advantage that with minor modifications, such as the size of the rubber contact, the device could potentially be applied to different anatomical locations, such as the footpad or the ankle, and in different disease models and species.

Results recorded by PAM were consistent, with no significant differences observed between the left and right LWTs of the non-injected knees in all the studies. During the pilot study in rats a large increase in the LWT of the un-injected limbs was observed from day 0 to day 1, following the induction of inflammatory arthritis. This increase was due to the fact that there was no habituation period and the day 0 reading was the first time the animals had been handled and introduced to the device. Following this pilot study in rats a one day habituation assessment was introduced prior to the drug study in rats, and this resulted in the increase in LWT from day 0 to day 1 being absent. Moreover the absolute values of the LWTs of non-injected limbs remained constant at ~1100gf over the 28 day study period. This is in contrast to a calibrated forceps study in which the LWT of the paw increases as the animal gains weight (Luis-Delgado et al. 2006).

PAM was able to detect an FCA-induced hypersensitivity in both mice and rats, observed as a decrease in the absolute LWT for the injected limb, alongside a decrease in the LWT ratio. Prednisolone (Pyne et al. 2004) is a gold standard used in drug screening that was used alongside celecoxib (Warner and Mitchell 2004) and morphine (Peloso et al. 2000; Caldwell et al. 2002) in the dosing studies, as drugs that are known to be clinically effective at relieving pain and hypersensitivity of arthritis. The highest doses of all three drugs

investigated caused a significant reduction in the joint hypersensitivity of FCA-injected animals when compared to vehicle.

In all studies the animals' weight distribution measurements were taken alongside PAM LWTs in order to compare the two readouts. The weight distribution ratio is a readout that measures how much vertical compression through the length of the limb that the knee joint can take, created by gravity acting downwards on the body weight of the animal and the fixed horizontal platform. The PAM LWT ratio measures how much horizontal compression across the width of the knee that the joint can take, created as a squeeze between the force transducer and forefinger. Both readouts utilise a mechanical stimulus, applied to the knee joint up until the point of becoming noxious eliciting a reflex withdrawal from the stimulus. In both pilot studies correlation graphs plotting the LWT ratio against the WD ratio showed strong positive correlations between the two measures. Furthermore the AUC graphs created from the results of the dosing period in the drug groups are very similar, with statistically significant reductions in the hypersensitivity of the FCA-injected knee joint being observed in the same groups in both readouts. These similarities suggest that PAM is capable of accurately detecting FCA-induced hypersensitivity of the knee joint and subsequently dose related, drug-induced reduction of this hypersensitivity.

Previous studies carried out in this laboratory used a subjective three point scoring system to rate joint hypersensitivity, with 0 being no different from naïve and 3 being the most sensitive, by squeezing the knee joint between the fingers until the animal withdrew its limb (Gauldie et al. 2004). This method was comparable to the Ritchie index used in the clinic to assess the tenderness in patients joints based on a four point scale (Ritchie et al. 1968). The clinical dolorimeter has regularly been used to evoke a mechanical squeeze to objectively assess joint tenderness in patients suffering from both osteoarthritis and fibromyalgia (Ottillinger et al. 2001; Harris et al. 2006). PAM now provides an objective

behavioural assessment for pre-clinical screening in conscious animals that is comparative with the clinical dolorimeter (Langley et al. 1983).

In conclusion, the studies presented in this chapter show that PAM, a novel behavioural tool for objectively assessing localised primary hypersensitivity, has been validated during two animal models of chronic inflammatory joint pain. Moreover, the device can be used in future studies, either to complement or replace existing readouts as a pre-clinical screening tool for assessing the efficacy of novel pharmacological compounds designed for treating chronic inflammatory joint pain.

Chapter 6

The role of Na_v1.7, Na_v1.8 and Na_v1.9 in FCA-induced chronic inflammatory joint hypersensitivity

6.1 Introduction

VGSCs are fundamental in regulating the excitability of neurons, and changes in the expression of these channels can lead to chronic pain (Waxman et al. 1999; Lai et al. 2004). There are nine sodium channel α -subunits (Goldin et al. 2000), and a great deal of research has focused on the role that the TTX-S $\text{Na}_v1.7$ and the TTX-R $\text{Na}_v1.8$ and $\text{Na}_v1.9$ isoforms, which are predominantly expressed in peripheral damage sensing neurones (Black et al. 1996; Fang et al. 2002; Djouhri et al. 2003a; b), play in both neuropathic and acute inflammatory pain. The expression of $\text{Na}_v1.7$, $\text{Na}_v1.8$ and $\text{Na}_v1.9$ has been shown to be highly dynamic in DRG tissue and their modulation may present a therapeutic opportunity for the selective manipulation of primary sensory neurons, as discussed in chapter 1. It has long been hypothesised that $\text{Na}_v1.7$, $\text{Na}_v1.8$ and $\text{Na}_v1.9$ all appear to have some role in either the establishment or maintenance of the hypersensitivity observed in models of acute (<7 days post-insult) inflammatory pain (Tanaka et al. 1998; Black et al. 2004; Gould et al. 2004; Nassar et al. 2004; Priest et al. 2005; Villarreal et al. 2005; Ekberg and Adams 2006). Numerous studies have been performed describing the long-term changes in the expression levels of these individual sodium channels during neuropathic pain as they are either up- or down-regulated following injury to the nervous system (for reviews see Lai et al. 2003; Amir et al. 2006; Rogers et al. 2006). Until this study little information existed on sodium channel expression in DRG tissue during a chronic (>7 days) model of pain following an inflammatory insult. One study had investigated the effect of intraplantar FCA on the expression of the entire sodium channel population up to 6 months post-insult, but did not provide details of changes in the individual channel isoforms (Gould et al. 1998).

Recent clinical studies have provided convincing evidence to suggest that the sodium channel $\text{Na}_v1.7$ plays a crucial role in the detection of pain and pain sensitivity. Families suffering from congenital indifference to pain (CIP), a condition characterised by absence of the ability to sense pain, all suffer from a mutation in the *SCN9A* gene that

encodes $\text{Na}_v1.7$ that leads to a complete loss of functionality of the channel (Cox et al. 2006; Ahmad et al. 2007; Goldberg et al. 2007). Conversely paroxysmal extreme pain disorder (Fertleman et al. 2006) and inherited erythromelalgia (Lee et al. 2007) are two autosomal dominant pain conditions where point mutations in the *SCN9A* gene cause hyperpolarising shifts in the voltage dependence of activation of the channel leading to increased neuronal excitability. These mutations cause severe rectal and facial pain, and chronic burning sensations in the hands and feet in the respective conditions.

Recent studies have shown that VGSC blockers displaying selectivity toward $\text{Na}_v1.8$ can provide significant analgesia in rat models of neuropathic and inflammatory pain (Ekberg et al. 2006; Jarvis et al. 2007). The μO -conotoxin peptide MrVIB extracted from cone snails reduced acute post incisional pain when injected into the footpad of male rats prior to making the incision (Bulaj et al. 2006), and intrathecal MrVIB attenuated the reduction in thermal and mechanical paw withdrawal thresholds following intra-plantar FCA injection in male rats (Ekberg et al. 2006). The molecule A-803467 is a potent and selective inhibitor of $\text{Na}_v1.8$ channels that reduced both mechanical and thermal hypersensitivity in rats subjected to a range of acute inflammatory insults (Jarvis et al. 2007).

$\text{Na}_v1.9$ null mutant mice showed reduced sensitivity to the inflammatory mediators FCA, prostaglandin E2 and carrageenan, but no change in pain behaviours in two models of neuropathic pain (Priest et al. 2005), supporting the suggestion that the $\text{Na}_v1.9$ isoform has a role in pain sensations produced from inflamed tissues.

6.1.1 Aims

The studies described in this chapter were designed and performed with the aim to better understand the role of $\text{Na}_v1.7$, $\text{Na}_v1.8$ and $\text{Na}_v1.9$ in the maintenance of chronic pain following inflammatory insult. Firstly, an immunohistochemistry study was designed to establish if there are any long term changes in the expression of $\text{Na}_v1.7$, $\text{Na}_v1.8$ and $\text{Na}_v1.9$

in L3-L5 DRGs up to 28 days during the FCA-induced unilateral inflammatory joint pain model (Wilson et al. 2006; Martindale et al. 2007). Moreover, by using the fluorescent retrograde tracer FB, this study was able to examine expression levels in a specific sub-population of cell bodies belonging only to the sensory neurons innervating the FCA-injected knee joint (Salo and Theriault 1997; Ivanavicius et al. 2004; Fernihough et al. 2005). In this way any subtle changes in channel expression could be directly attributed to the sensory neurones innervating the inflamed joint. It is hypothesised that an up-regulation of these sodium channels in the sensory afferent nerves innervating the knee joint could lead to a neuronal hyperexcitability that manifests itself as a chronic joint pain.

The second aim was to investigate any behavioural analgesic effects of the sodium channel blockers; ralfinamide (Veneroni et al. 2003; Cattabeni 2004; Stummann et al. 2005; Yamane et al. 2007) and a compound synthesised at GlaxoSmithKline which is selective for $\text{Na}_v1.8$ at low doses, and is referred to as Compound A, when dosed directly into the FCA-injected knee on days 14-18 of the same model of inflammatory pain. Injecting the compounds directly into the knee joint space allowed the dose of drug present at the channels to be accurately calculated. Any effects of the drug could be attributed to action on channels at the site of inflammation in the periphery and not through any centrally mediated effects. Three doses of each test drug were carefully selected to target the sodium channels present in the sensory afferent nerves to block the transmission of signals in nociceptors without blocking low threshold pressure and touch receptors or motor neurones.

6.2 Methods

Two studies investigating the role of sodium channels in FCA-induced chronic inflammatory joint pain are described. Firstly IHC was performed at GlaxoSmithKline in order to investigate $\text{Na}_v1.7$, $\text{Na}_v1.8$ and $\text{Na}_v1.9$ sodium channel expression in L3-5 DRGs of 54 adult male Wistar rats (150-200g, Charles River, UK). Secondly a behavioural study was

performed at The University of Edinburgh investigating the effect of sodium channel blocking drugs administered directly into the knee joint in 56 adult male Wistar rats (150-200g, Charles River, UK). The behavioural study was assisted throughout by Miss Fiona Moultrie, an intercalated BSc Hons project student. All animals were housed four to a cage in a 12 hour dark/light cycle and were given access to water and standard animal feed *ad libitum*. All animals were allowed at least five days acclimatisation prior to behavioural testing, followed by a week of habituation to the behavioural tests.

6.2.1 Design of IHC studies investigating sodium channel expression

6.2.1.1 Assessment of weight distribution

The distribution of a rat's body weight (WD) across their hind limbs was measured using the incapacitance tester as described in section 2.2.1. Following the initial WD assessment, all animals (n=54) received an injection of 10µl of 2% FB into the knee joint under brief halothane anaesthesia (see section 2.4.1). Seven days after the injection of FB, animals were divided into those which then received FCA (n=24) or control animals that were anaesthetised but received no injection (n=30). Following the anaesthetic a single control group of animals was killed to provide the day 0 tissues (n=6). FCA-treated rats were then killed in groups on days 7, 14, 21 or 28 post-FCA (n=6) and at the same time points the corresponding control groups (n=6) were also killed. Animals were killed using a rising concentration of CO₂ followed by cervical dislocation.

6.2.1.2 Immunohistochemistry techniques

Ipsilateral dorsal root ganglia (DRGs) were removed from levels L3, L4 and L5 in all study animals immediately after confirmation of death. They were embedded in optimum cutting temperature (TissueTek, Canada) compound and snap frozen in isopentane cooled on

dry ice. DRGs were carefully labelled, wrapped in tin foil and then stored at -80°C until they were sectioned. Full details of the tissue processing and sectioning can be found in section 2.4.2. Every 10th section was counted, so that sections at a distance of 120µm apart were counted to avoid counting the same cell body twice.

A pilot study using a range of concentrations of the sodium channel primary antibodies Na_v1.7, Na_v1.8 and Na_v1.9 was performed in order to establish the optimum concentration required to achieve the best signal to background fluorescence. After the working concentration of all three primary antibodies was decided, the main immunohistochemical time-course study was performed. In all cases the optimum primary antibody concentration was 1:200. Slides were prepared and treated with the specific sodium channel primary antibody and secondary antibodies as described in section 2.4.3. Full details of the primary and secondary antibodies used are given in section 2.4.5. Fluorescence in DRGs was observed and quantified as described in section 2.4.7.

6.2.2 Ralfinamide and Compound A behaviour study design

All study animals (n=56) were given two days of behavioural habituation before the study began. Behavioural tests comprised of measuring the WD of the animals and recording the LWT of the animals to a knee joint squeeze using the PAM device as previously described in section 2.2.1. and 2.2.2, and validated in chapter 5. The order in which the behavioural measurements were made was designed in order to give the animals breaks between measurements and prevent them becoming stressed by the procedures. Initially three consecutive weight distribution readings were taken and then the animal was returned to their cage. Five minutes later, the animal had alternate measurements of the FCA-injected and contralateral limb assessed using PAM. This was repeated three times with a one minute break in between each measurement. Finally the animal's knee joint diameter and body weight were measured.

On day 0 behavioural assessments were made and animals received an intra-articular injection of 150 μ g of FCA into the left (ipsilateral) knee joint under transient anaesthesia as described in section 2.1.2. Subsequent behavioural tests were then made on days 1, 3, 7, 9 and 11 post-FCA. On day 11 animals were ranked in descending order based on their PAM behavioural score for that day and randomised into 7 groups (n=8) to ensure an even spread of sensitivity of animal in each dosing group. Blind drug dosing took place on days 14, 16 and 18 post-FCA. Rats were transiently anaesthetised (3% halothane in O₂ at 1.5L.min⁻¹) and 100 μ l of drug or vehicle was injected into the knee joint space using a 25-gauge needle (BD Microlance, UK). Behavioural assessments were performed one hour post-injection when the animals had fully recovered from anaesthesia, and 24 hours later on non-dosing days (15 and 17 post-FCA). Further behavioural assessments were made on day 21 to observe any long term effects of the drugs dosed. Animals were killed at the end of the study using a rising concentration of CO₂ followed by cervical dislocation.

6.2.2.1 Drugs

Ralfinamide (made in house at GlaxoSmithKline, MW= 398.5), and Compound A (made in house at GlaxoSmithKline, MW= 430.6), were dissolved in 2% DMSO (Fisher, UK), 66% PEG400 (Sigma, UK) and 32% distilled water. 100 μ l of drug was injected into the knee joint cavity and was immediately diluted 1:1 by the 100 μ l of fluid already present in the arthritic knee (Barton 2007a). The final concentrations of the drug within the knee joint were ralfinamide at 10 μ M, 30 μ M and 100 μ M and Compound A at 200nM, 500nM and 1000nM.

The doses of ralfinamide investigated were chosen based on previously published data and information generated in house at GlaxoSmithKline (oral communications, Dr. A.J. Reeve and Dr. P. Green). Patch clamp studies in dorsal root ganglion neurons held at -70mV showed ralfinamide to have an IC₅₀ of 55 μ M and 22 μ M at the TTX-resistant and an TTX-sensitive sodium channels respectively (Stummann et al. 2005). Other studies revealed that

ralfinamide had an IC_{50} of $22\mu M$ in human $Na_v1.8$ channels held at $-80mV$ (Dekker et al. 2005). A significant analgesic effect of the drug has been shown in behavioural studies, with 1 and $3mg.kg^{-1}$ administered either intra-peritoneally or orally resulting in the attenuation of the mechanical allodynia observed in the paw of FCA-treated rats (Veneroni et al. 2003). These doses of 1 and $3mg.kg^{-1}$ given to a $250g$ rat with an estimated blood volume of $17.5ml$ (7% of the body weight) were estimated to give concentrations of approximately $35\mu M$ and $105\mu M$ respectively.

Ideally a behavioural test (e.g., rotarod) investigating any motor effects or ataxia caused by the drug would have been performed but due to licence restrictions this was not possible. The doses of ralfinamide effective in the behavioural test described above did not impair the gross motor function, and *in vitro* evidence suggests that ralfinamide causes a frequency-dependent block selectively on nociceptive afferents (Veneroni et al. 2003). Furthermore, data generated in house by GlaxoSmithKline showed that ralfinamide at a dose of $10mg.kg^{-1}$ had no motor effect when tested on either a rotarod or in the LABORAS apparatus (Jones and Roberts 1968; Quinn et al. 2003).

The doses selected for the Compound A were based on in-house data provided by GlaxoSmithKline. An oral dose of $3mg.kg^{-1}$ caused a significant analgesic effect in FCA-induced joint pain. Furthermore, *in vitro* data indicated the compound is selective for $Na_v1.8$ at $158nM$, and both $Na_v1.8$ and $Na_v1.3$ at $500nM$.

6.2.3 Statistical analysis

The value of cells expressing the sodium channel of interest were graphically expressed as a group mean (\pm SEM) percentage of the FB population on days 7, 14, 21 and 28. Statistical analysis comparing the FCA injected group of animals with time matched control groups was performed on each individual day using a Mann-Whitney test. A two-way ANOVA was used to compare the FCA and time matched control groups at all time

points over the whole study. Changes within the FCA or control groups over time were compared using a Kruskal-Wallis ANOVA. In all cases a P value of ≤ 0.05 was considered statistically significant.

Behavioural results were expressed as the group mean (\pm SEM) for the WD and the LWT ratios. The statistical analysis used to compare differences within the same group over time was the one-way ANOVA followed by a Dunn's post-hoc test. The test used to compare different groups of animals, such as the dosing groups, over time was the two-way ANOVA followed by Bonferroni's post-hoc test. In order to compare statistically the AUC values calculated for each drug over the dosing period, a Kruskal-Wallis ANOVA was used followed by Dunn's post-hoc test comparing the dosing groups back to vehicle. In all cases a P value of ≤ 0.05 was considered statistically significant.

6.3 Results

6.3.1 IHC channel expression study results

6.3.1.1 Assessment of weight distribution

The injection of FB into the left knee joint did not change how the animals distributed their body weight over their hind limbs. The mean (\pm SEM) WD value on day 0 before the injection was 1.01 ± 0.04 and was not significantly different from the mean WD value on day 7, namely 0.94 ± 0.05 ($P > 0.05$, Wilcoxon, $n=54$; Figure 6.1).

Injection of $150\mu\text{l}$ of FCA into the left knee joint caused a decrease in the WD ratio as animals shifted their stance and carried less weight on the injected limb. The day 7 mean WD value prior to injection of FCA was 0.96 ± 0.03 and this fell significantly to 0.37 ± 0.03 by day 14 ($P < 0.05$, Wilcoxon, $n=24$; Figure 6.1). The FCA-injected animals continued to show a significantly lower group mean WD ratio at all time points post-FCA compared to

the control animals ($P<0.05$, Kruskal-Wallis ANOVA, $n=54$). There was no significant change in the WD ratio within the control animals at any time.

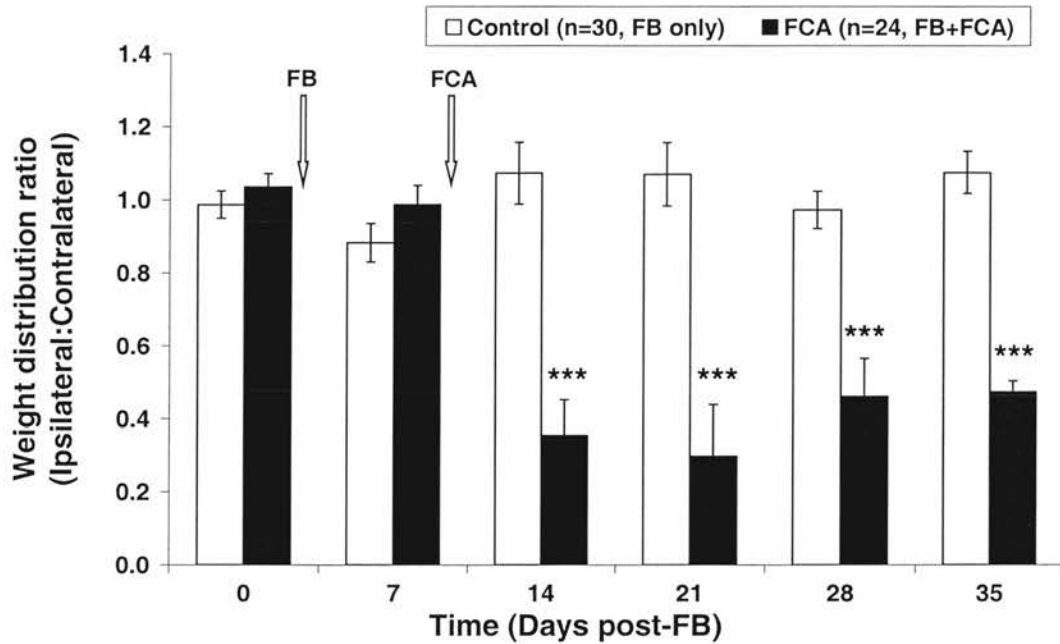


Figure 6.1 Graph showing the changes in weight distribution of the 35 day study following injection of FB (day 0) and FCA (day 7) into the left (ipsilateral) knee joint. FB caused no significant difference in the mean weight distribution ($P>0.05$, comparing the day 0 ratio to the day 7 ratio, using a Wilcoxon test, $n=54$). FCA injected into the ipsilateral knee seven days later caused a significant reduction in the weight distribution ratio at all time point's post-FCA, as the rats shift their body, to place less weight through the ipsilateral limb. * represents statistical significance ($P<0.05$) comparing the WD ratio of the FCA injected animals to that of control using a Kruskal–Wallis ANOVA followed by Dunn's post-hoc test.

6.3.1.2 Fast Blue fluorescence in DRG cell bodies

Intra-articular injection of FB into the knee joint resulted in certain individual cell bodies fluorescing in the DRG (Figure 6.2). During this study 162 DRGs were harvested and between 4 and 6 sections of each DRG were observed under the microscope. A total of 2463 FB fluorescing cell bodies were counted within these sections, with an average of 5 (min=0, max=28) fluorescing cells being identified per individual ganglion. There was no significant difference in the group mean number of FB positive cells counted in animals within the FCA-injected group compared to the control group ($P>0.05$, 28 ± 2 vs. 32 ± 2 ; Mann Whitney). Of the 2463 FB positive cells counted, 44% were observed in L3, 38% in L4 and

the remaining 18% in L5. Brightly fluorescing satellite cells surrounding FB-positive cell bodies were observed throughout the study at each time point.

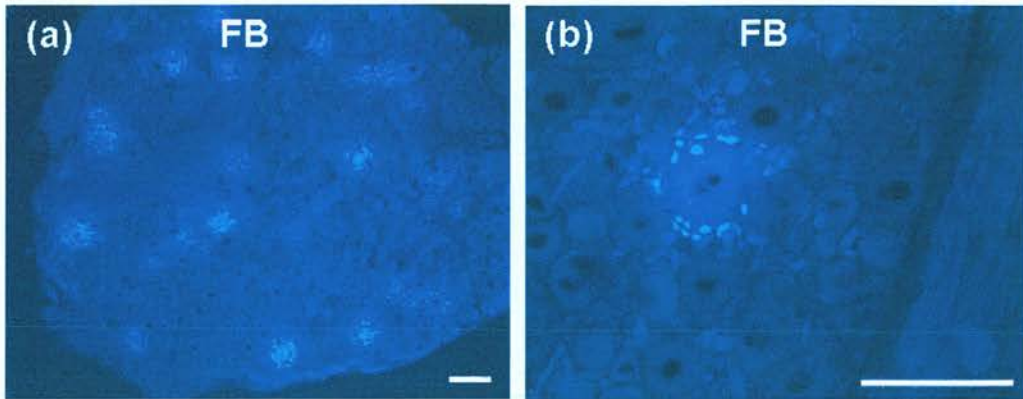


Figure 6.2 Representative photomicrographs showing FB fluorescence in FCA-injected L3-5 DRG tissue 14 days post-FCA. (a) A small proportion of the total population of DRG cell bodies were FB positive and are easily identifiable from the non-fluorescing cells. (b) A higher magnification illustrates that surrounding the FB positive cell bodies are smaller satellite cells that are also brightly fluorescing. Scale bar = 100µm.

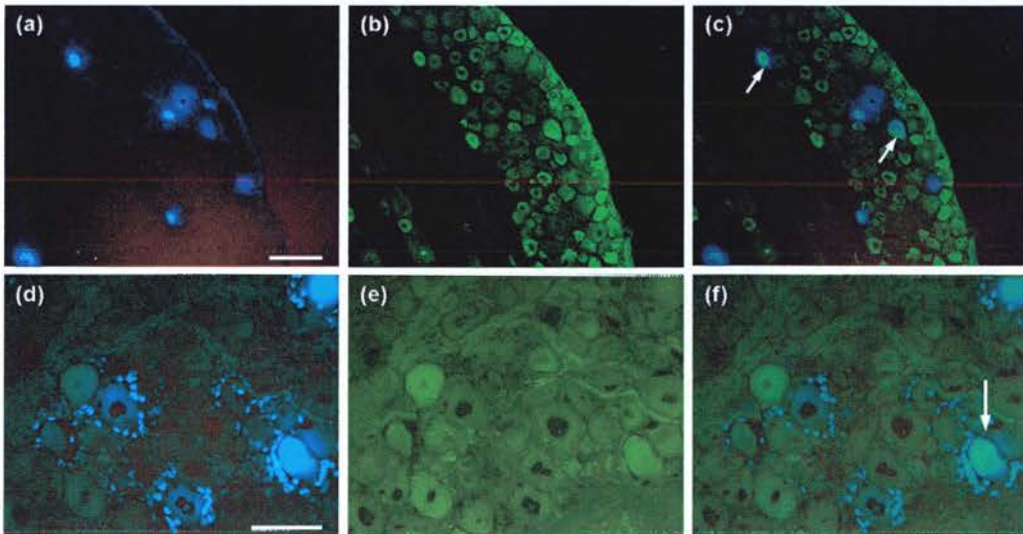


Figure 6.3 Representative photomicrographs of two different DRG sections (a–c) and (d–f) taken from animals 14 days post-FCA. Images captured show (a) and (d) the FB fluorescence, (b) and (e) the NaV1.7 positive cells, (c) and (f) an overlay of the FB and NaV1.7 images. Arrows indicate cells positive for both the FB and NaV1.7 channel. Note that the FB positive cells were not always necessarily positive for the sodium channel and vice versa. By switching between filters without moving the sections it was possible to count all the cells that were positive for both. Scale bar = 100µm, applies to each row.

6.3.1.3 Fast Blue and Na_v channel co-localisation

By flicking between the filters on the microscope to allow different excitation frequencies of light to be shone on the sections, the fluorescence of both FB positive and sodium channel positive cells bodies were observed within the same section (Figure 6.3a, b, d and e). Absence of bleed through between filters was confirmed by the presence of three types of cell, namely cells that were only positive for one of the sodium channels, cells that were only positive for FB, and cells that were positive for one of the sodium channels and also for FB.

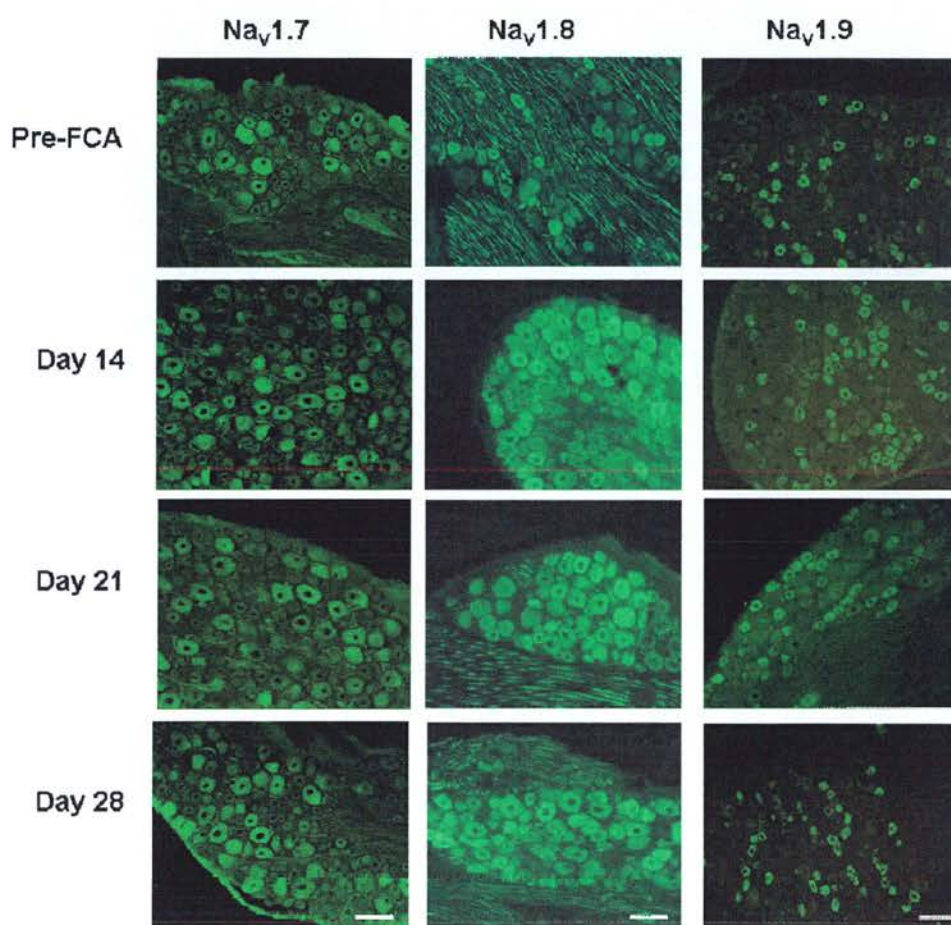


Figure 6.4 Representative images of $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$ immunofluorescence in FCA-injected ipsilateral DRG sections before administration of FCA and on days 14, 21 and 28 post-FCA. It is not clear from simple visual inspection whether expression levels change over time, highlighting the need for quantification. Scale bar = 100 μ m and applies to each column.

6.3.1.4 Changes in Na_v1.7 channel expression

The effect of FCA or control treatment on Na_v1.7 expression in knee joint afferent cell bodies was assessed on days 7, 14, 21 and 28, post-FCA (Figure 6.4). FCA caused an increase in the percentage of Na_v1.7 positive cells in the FB positive DRG population of L3-L5 (Table 6.1). The mean group percentage (\pm SEM) of FCA animals was significantly higher than control animals on days 14 and 28 post-FCA ($P<0.05$, Mann Whitney, control vs. FCA; Figure 6.5a). In control animals no significant change was observed in the group mean percentage at any time point over the 28 day period ($P>0.05$, Kruskal-Wallis ANOVA). A total of 508 FB-positive cells were counted in the 5 control groups and 385 in the 4 FCA groups. When all the time points for both FCA-treated and control animals were considered together the mean percentage of Na_v1.7 positive cells in FCA-injected animals was significantly higher than that in all the control animals ($P<0.01$, FCA vs. control, $42.2\% \pm 1.7$ vs. $32.3\% \pm 2.3$).

Day (post-FCA)	Control (n=30)		FCA-injected (n=24)	
	Mean %	SEM	Mean %	SEM
0	27.7 (28/102)	3.3	-	-
7	35.8 (41/115)	6.1	35.5 (28/79)	3.9
14	32.3 (33/101)	3.4	↑ 45.9 (56/122)	2.7
21	36.0 (33/92)	3.1	42.9 (39/92)	3.1
28	25.4 (25/98)	4.0	↑ 47.8 (44/92)	3.9

Table 6.1 The group mean percentage of FB positive cells expressing the Na_v1.7 channel in L3-5 DRG of control and FCA-injected left knee joints. Numbers within the brackets indicate the number of Na_v1.7 positive cells in the FB population (Na_v1.7 / FB). Arrows indicate the time points at which FCA caused an increase ($P<0.05$, Mann Whitney) in the percentage of FB cells that are Na_v1.7 positive when compared to control. The day zero values were taken as a basal reading from animals prior to FCA or control treatment and therefore only one group (n=6) was required as the animals had not yet been separated into treatment groups.

6.3.1.5 Changes in Na_v1.8 channel expression

Following FCA or no control the expression of Na_v1.8 in FB positive cell bodies of ipsilateral L3–L5 DRGs was assessed on days 7, 14, 21 and 28, post-FCA (Figure 6.4). There was a significantly higher percentage of Na_v1.8 expressing cells in the FCA-injected group compared to control on days 14 and 28 ($P<0.05$, Mann Whitney, Table 6.2). No significant change was observed in the group mean percentage of control animals at any time point over the 28 day period (Figure 6.5b, $P>0.05$, Kruskal-Wallis ANOVA). A total of 417 FB positive cells were counted in the 5 control groups and 365 in the 4 FCA groups. The mean percentage of all Na_v1.8 positive cells at all time points in the FCA-injected set of animals was significantly higher than that in the control groups (Mann Whitney, $P<0.01$, $49.8\% \pm 2.2$ vs. $38.9\% \pm 1.1$, FCA vs. control).

Day (post-FCA)	Control (n=30)		FCA-injected (n=24)	
	Mean %	SEM	Mean %	SEM
0	37.3 (28/75)	4.4	-	-
7	37.5 (50/133)	2.0	41.6 (31/74)	4.8
14	38.0 (41/108)	2.3	↑ 55.0 (44/80)	2.6
21	39.9 (26/64)	2.8	48.4 (40/82)	4.4
28	40.8 (43/105)	2.0	↑ 53.2 (33/62)	4.7

Table 6.2 The group mean percentage of FB positive cells expressing the Na_v1.8 channel in L3-5 DRG of control and FCA-injected left knee joints. Arrows indicate the time points at which FCA caused an increase ($P<0.05$, Mann Whitney) in the percentage of FB cells that are Na_v1.7 positive when compared to control. The group mean percentages were calculated from counting the FB cells in 4-6 sections in each of the three DRG taken from the six animals in the group (n=6).

6.3.1.6 Changes in Na_v1.9 channel expression

The effect of FCA or control treatment on Na_v1.9 expression in knee joint afferent cell bodies of L3–L5 was assessed on days 7, 14, 21 and 28, post-FCA (Table 6.3). FCA-injected animals have a significantly higher group mean percentage (\pm SEM) of Na_v1.9

positive cells expressed in the FB population compared to control animals at days 14, 21 and 28 ($P < 0.05$, Mann Whitney, Figure 6.5c). No significant change was observed in the group mean percentage at any time point over the 28 day period in control animals ($P > 0.05$, Kruskal-Wallis ANOVA). A total of 479 FB positive cells were counted in the control groups and 309 in the FCA groups. The mean percentage (\pm SEM) of Na_v1.9 positive cells in all the FCA-injected animals ($45\% \pm 2.2$) was significantly higher ($P < 0.001$) than that in all the control animals ($24.7\% \pm 1.9$) when every time point was taken into account.

Day (post-FCA)	Control (n=30)		FCA-injected (n=24)	
	Mean %	SEM	Mean %	SEM
0	32.7 (23/70)	6.8	-	-
7	24.9 (42/169)	3.1	39.2 (29/74)	4.7
14	22.7 (23/101)	3.7	↑ 46.4 (36/77)	3.3
21	26.2 (15/57)	5.2	↑ 47.0 (47/100)	5.6
28	25.3 (21/82)	3.7	↑ 47.8 (34/71)	3.9

Table 6.3 The group mean percentage (\pm SEM) of FB positive cells expressing the Na_v1.9 channel in L3-5 DRG of control and FCA-injected left knee joints. Arrows indicate the time points at which FCA caused a significant increase ($P < 0.05$, Mann Whitney) in the percentage of FB cells that are Na_v1.9 positive when compared to control. The group mean percentages were calculated from counting the FB cells in 4-6 sections in each of the three DRG taken from the six animals in the group (n=6).

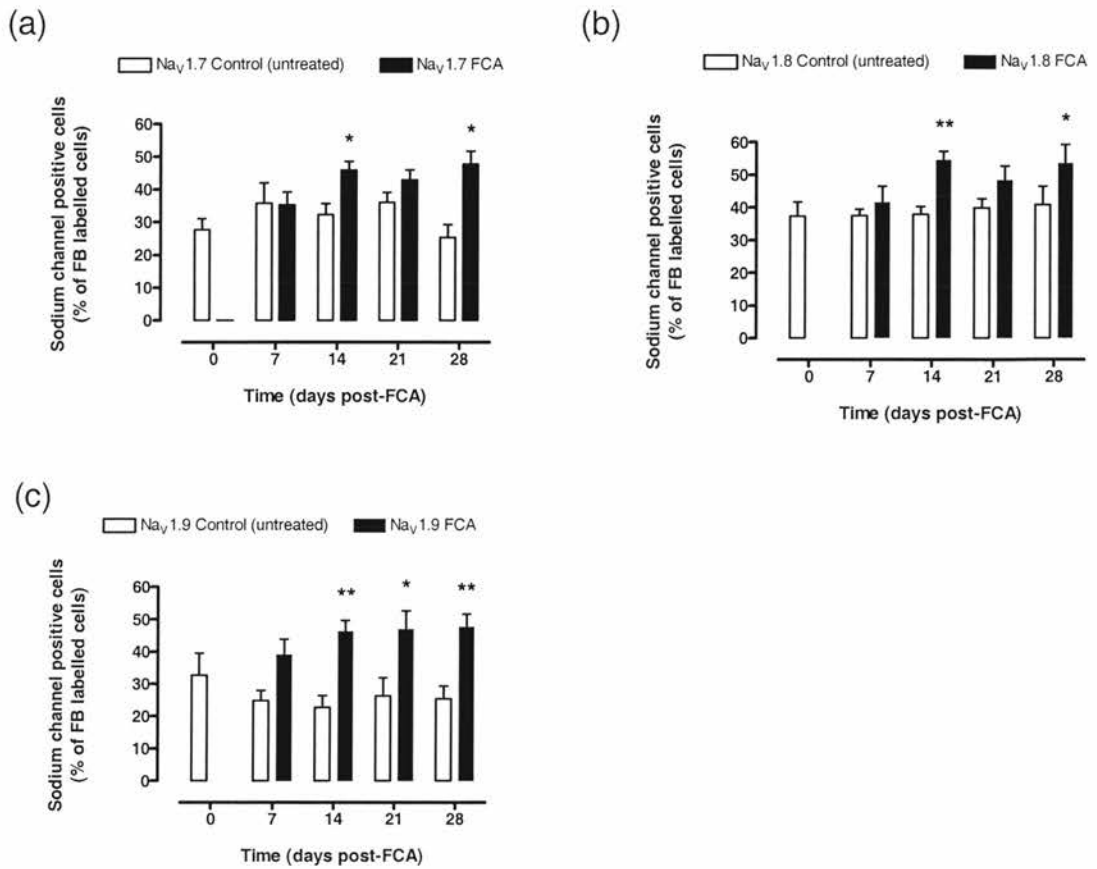


Figure 6.5 Quantitative analysis showed that the percentage of: (a) Na_v1.7, (b) Na_v1.8 and (c) Na_v1.9 immunofluorescent cells within the FB labelled DRG population are significantly upregulated in FCA treated animals, as compared with control animals at various points during the FCA time-course (Mann Whitney, * $P < 0.05$, ** $P < 0.01$, $n=6$). Note that at 7 days post-FCA there is no significant difference in FCA treated animals and control animals with regards to all the Na_v channels, and further no significant difference at day 21 when looking at Na_v1.7 and Na_v1.8 channels.

6.3.2 Ralfinamide and Compound A behaviour study results

6.3.2.1 Body weight and diameter of the knee joint

FCA or the test drugs injected into the knee joint had no adverse affect on the general health or motor function of the animals. Animals were anaesthetised four times during the study, and on two occasions an animal suffered an adverse reaction and died under anaesthesia. Where appropriate the data generated for these animals is included up to the point at which they died. The mean weight of the animals prior to injection of FCA was 209 ± 1 g, a value that decreased insignificantly to 207 ± 1 g on the next day ($P > 0.05$,

Wilcoxon, $n=56$). Following this slight decrease, rats gained weight on a daily basis throughout the study (Figure 6.6). There was no significant difference in the mean body weight of any of the dosing groups at any time point in the study ($P>0.05$, two-way ANOVA).

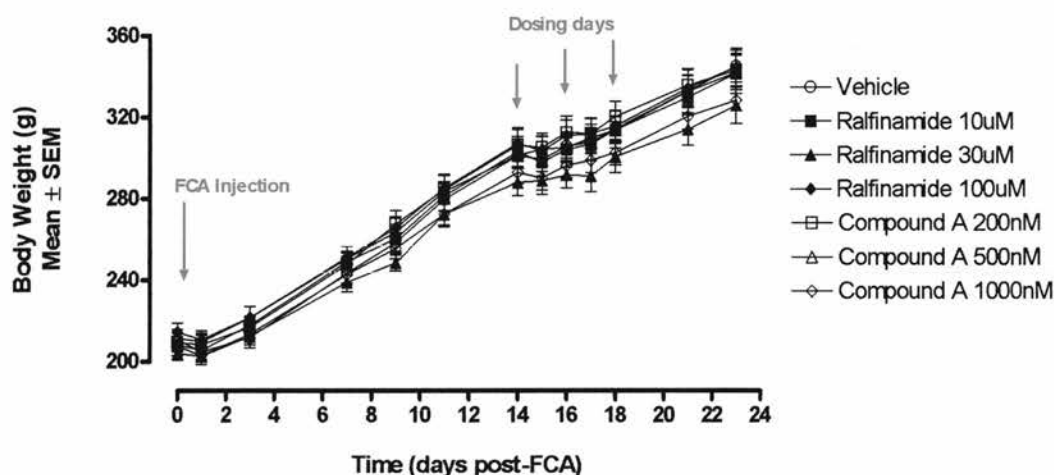


Figure 6.6 Plot of the body weight against time showing the effect of FCA (150 μ g, intra-articular), ralfinamide (100 μ l, 10 μ M and 100 μ M) and Compound A (100 μ l, 200nM and 1000nM) on the body weight of the rats. There were no significant differences in the mean body weight between any of the dosing groups ($P>0.05$, two-way ANOVA).

Inflammation induced by FCA resulted in the knee joint diameter ratio to decrease significantly from 1.03 ± 0.01 prior to injection, to 0.72 ± 0.01 , 24 hours after the injection ($P<0.05$, Wilcoxon, $n=56$ Figure 6.7). This change in ratio resulted from the FCA-treated joint diameter increasing significantly from 9.00 ± 0.03 mm to 13.05 ± 0.1 mm during this time ($P<0.05$, paired t-test, $n=56$), whilst the contralateral limb remained unchanged. During the ten day period from day 1 to day 11 post-FCA, the knee joint diameter ratio gradually began to return towards the pre-injection value of 1, as the swelling in the ipsilateral knee slowly began to resolve. On day 11 the joint diameter ratio of all the animals was 0.90 ± 0.01 . This value was still significantly lower than the day 0 value, but was also significantly higher than the day 1 value ($P<0.05$, one-way ANOVA, $n=56$, Figure 6.7b). The knee joint

diameter ratio did not significantly change after day 11, for the rest of the study ($P>0.05$, two-way ANOVA, $n=54$, Figure 6.7a).

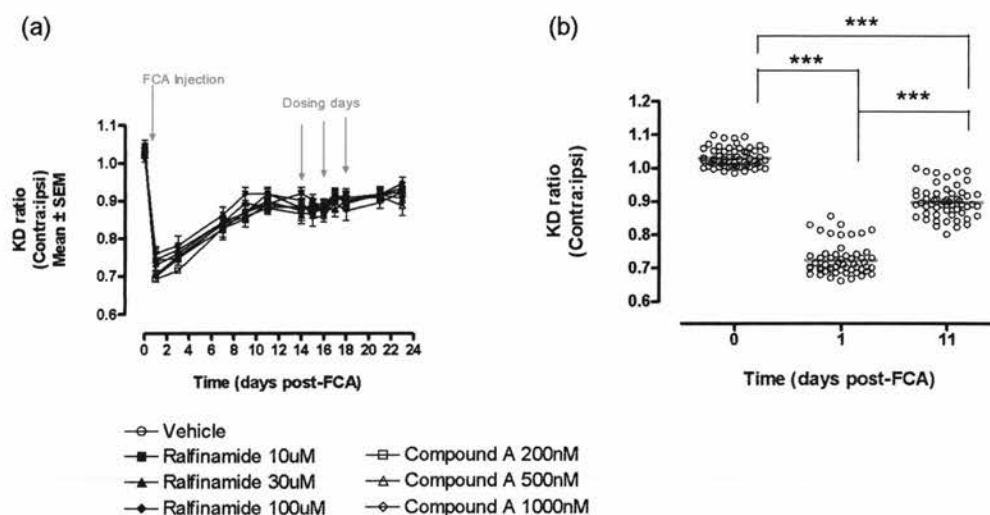


Figure 6.7 FCA caused the KD ratio to fall significantly from day 0 to day 1 ($P<0.05$, Wilcoxon, $n=56$). (a) The KD ratio then increased daily from day 1 to day 11. (b) On day 11 the KD ratio was still significantly lower than the day 0 value, but also significantly higher than the day 1 value ($P<0.05$, one-way ANOVA, $n=56$). After day 11 the KD ratio did not significantly change during the dosing period (day 14-18 post-FCA) and up till the conclusion of the study ($P>0.05$, two-way ANOVA, $n=56$).

6.3.2.2 Assessment of weight distribution on days 0-11 post-FCA

FCA caused a reduction in the weight distribution ratio on all days up to day 11 as the rats altered their stance to place less weight through the FCA-treated left hind limb (Figure 6.8a). The mean day 0 ratio fell from 0.97 ± 0.02 , where animals carried an average of 71 ± 1 g on their left limb and 73 ± 1 g on their right limb, to a day 1 value of 0.18 ± 0.02 , where animals only carried 20 ± 1 g on the FCA-treated limb and 111 ± 2 g on the untreated limb. The decrease in WD ratio from day 0 to day 1 was statistically significant and remained so through to day 11 where the WD ratio was 0.25 ± 0.01 ($P<0.05$, one-way ANOVA, $n=56$, Figure 6.8b). At no time from day 0 to day 11 was there any significant difference between the mean WD ratio of animals that were subsequently to receive ralfinamide, Compound A or vehicle on days 14-18 post-FCA.

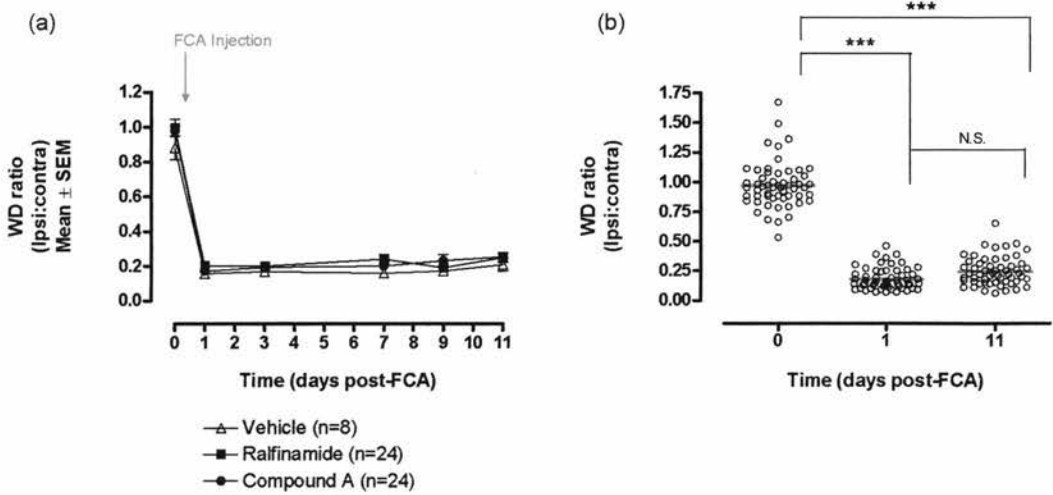


Figure 6.8 Graphs showing that (a) there was no significant difference in the mean group WD ratio of animals in the vehicle, ralfinamide or Compound A dosing groups at any time on days 0-11, prior to receiving treatment ($P < 0.05$, two-way, ANOVA, $n = 56$). (b) FCA into the left knee joint resulted in the WD ratio to decrease significantly from the day 0 value on days 1 and 11 post injection ($P < 0.05$, one-way ANOVA, $n = 56$).

6.3.2.3 Assessment of LWT on days 0-11 post-FCA

FCA reduced the basal LWT ratio on all test days up to day 11 as the injected limb became more sensitive to the squeeze applied and the rats withdrew the limb at a lower force (Figure 6.9a). The mean ratio on day 0 was 1.02 ± 0.01 , with the withdrawal threshold of the injected and non-injected limb being an average of $893 \pm 10\text{g}$ and $884 \pm 11\text{g}$ respectively. Following FCA the mean ratio fell to 0.35 ± 0.02 , with the withdrawal threshold of the injected and non-injected limb being an average of $248 \pm 7\text{g}$ and $716 \pm 11\text{g}$ respectively. The decrease in LWT ratio from day 0 to day 1 was significantly reduced and remained so until day 11, when the ratio was 0.52 ± 0.01 ($P < 0.05$, one-way ANOVA, $n = 56$, Figure 6.9b). At no time from day 0 to day 11 was there any significant difference between the mean LWT ratio of animals that were to receive ralfinamide, Compound A or vehicle on days 14-18 post-FCA.

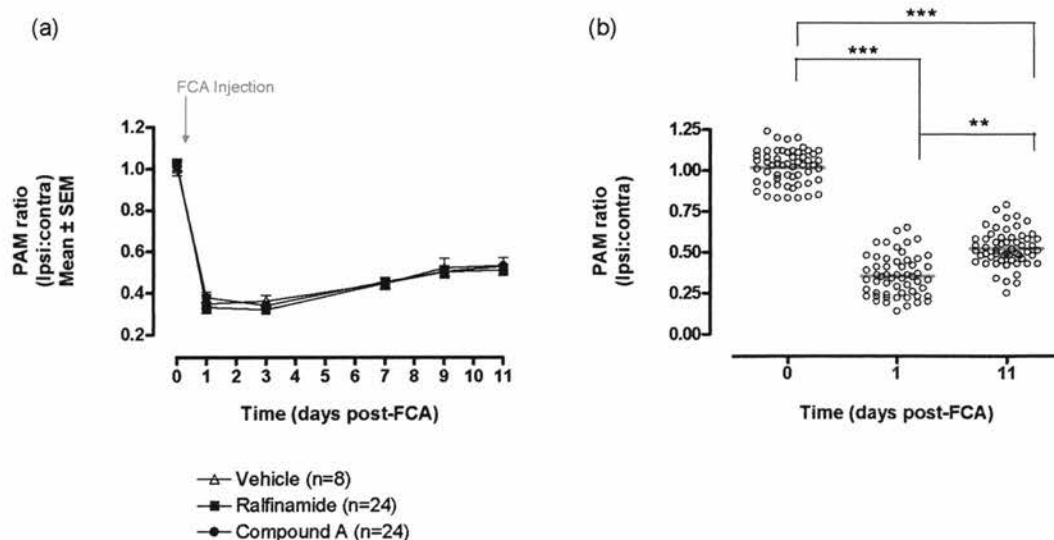


Figure 6.9 Graphs showing (a) the plot of PAM LWT ratio against time following FCA injection, there was no significant difference in the mean group PAM LWT ratio of animals in the vehicle, ralfinamide or Compound A dosing groups at any time on days 0-11 ($P < 0.05$, two-way, ANOVA, $n = 56$). (b) Injection of FCA into the left knee joint caused the PAM LWT ratio to decrease significantly from the day 0 value on days 1 and 11 post injection ($P < 0.05$, one-way ANOVA, $n = 56$).

6.3.2.4 Assessment of weight distribution on days 11-21 post-FCA

Following the establishment of an FCA-induced unilateral joint hypersensitivity, animals were dosed directly into the knee joint under brief anaesthesia with either ralfinamide (10 μ M, 30 μ M or 100 μ M), Compound A (200 nM, 500 nM or 1000 nM) or vehicle (2% DMSO, 66% PEG400 and 32% dH₂O). Dosing had no significant effect on the group WD ratio at any time point when compared to vehicle ($P > 0.05$, two-way ANOVA, $n = 8$; Figure 6.10a and 6.10c). Despite there being no significant effect of the drugs compared to vehicle, there did appear to be a trend that 100 μ M of ralfinamide consistently caused an increase in the WD ratio on the days it was dosed. This trend was also evident when examining the calculated AUC values. Despite the dose-related increase in WD ratio AUC values following ralfinamide, there was no significant difference in the AUC values of any dose of Compound A when compared to vehicle ($P > 0.05$, Kruskal-Wallis ANOVA, $n = 8$, Figure 6.10b). The range of doses of Compound A administered had no effect on the WD

ratio AUC values, and were not significantly different when compared to vehicle ($P>0.05$, Kruskal-Wallis ANOVA, $n=8$, Figure 6.10d).

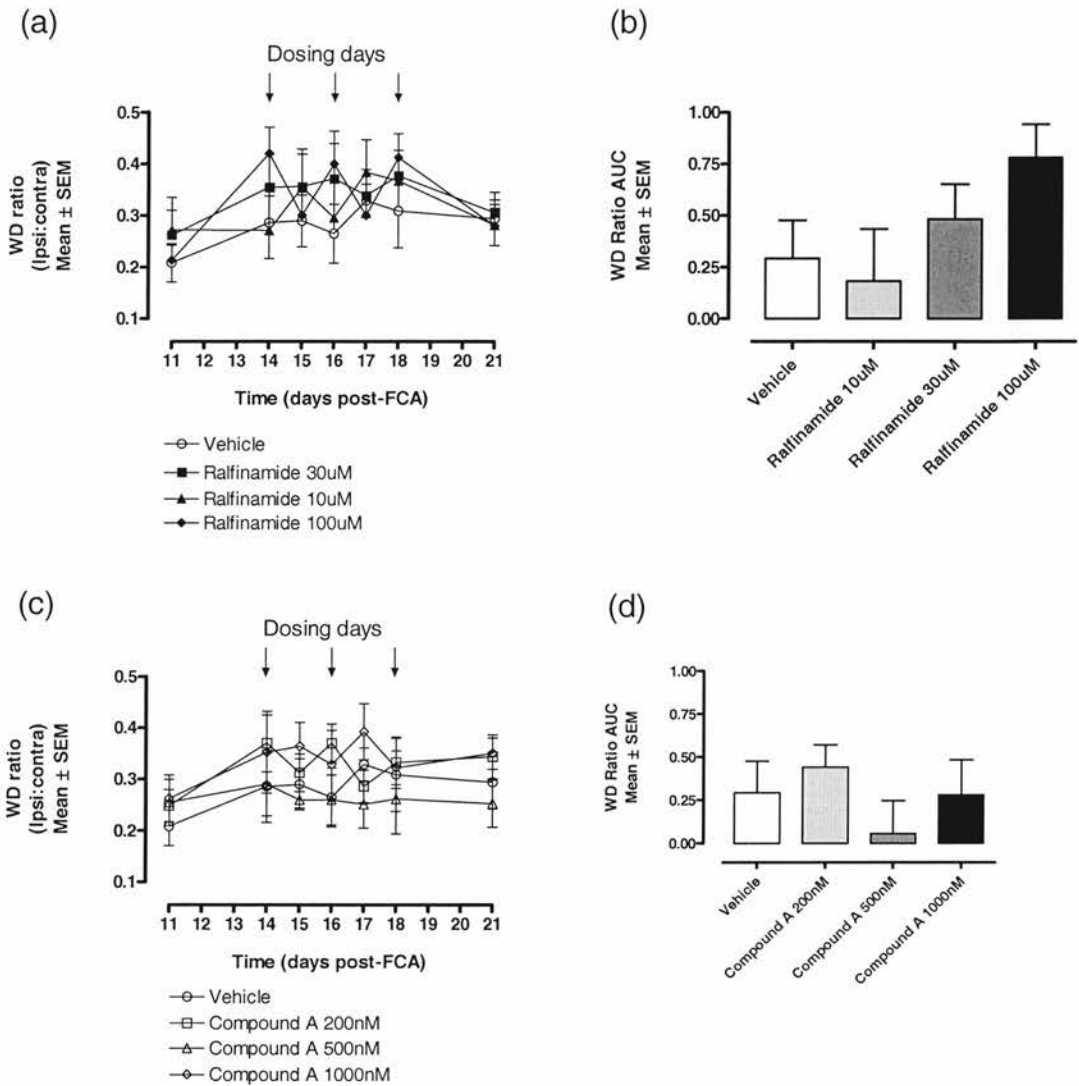


Figure 6.10 Graphs showing the effect of dosing (a) ralfinamide and (c) Compound A directly into the knee joint on days 14, 16 and 18, on the WD ratio. There was no significant difference in the group mean WD ratio of the three doses of either ralfinamide or Compound A at any time point compared to vehicle ($P>0.05$, two-way ANOVA, $n=8$; Figure 6.10a and 6.10c). Calculating the AUC for each group during the dosing period (14-18 post-FCA) allowed the cumulative effect of each drug to be calculated. There was no significant difference in the AUC values calculated from the three doses of either (b) ralfinamide or (d) Compound A compared to vehicle ($P>0.05$, Kruskal Wallis ANOVA, $n=8$).

6.3.2.5 Assessment of LWT on days 11-21 post-FCA

Neither Ralfinamide (10 μ M, 30 μ M or 100 μ M) nor Compound A (200nM, 500nM or 1000nM) had any significant effect on the group LWT ratio at any time point when compared to vehicle ($P>0.05$, two-way ANOVA, $n=8$; Figure 6.11a and 6.11c). Despite an absence of effect with the drugs relative to vehicle, there did appear to be a trend towards an increase in LWT ratio in the group of animals receiving 100 μ M of ralfinamide. This trend was also observed when examining the AUC values calculated for each dose, as there appears to be a dose-related increase in the LWT ratio AUC values. Despite this trend there was no statistically significant difference in the AUC values of any dose of ralfinamide administered, in comparison with the vehicle treated group ($P>0.05$, Kruskal-Wallis ANOVA, $n=8$, Figure 6.11b). Compound A in the doses studied, had no effect on the LWT ratio AUC values, and were not statistically different when compared to vehicle ($P>0.05$, Kruskal-Wallis ANOVA, $n=8$, Figure 6.11d).

6.3.2.6 Further analysis of the response to 100 μ M ralfinamide

The animals dosed with 100 μ M of ralfinamide showed a trend towards resolving the FCA-induced joint hypersensitivity as evidenced by increases in both the WD and LWT ratio values towards the normal value of 1. The weight distribution assessment of animals in this group showed that on the dosing days 14, 16 and 18 the mean WD ratio was significantly higher than the day 11 value ($P<0.05$, one-way ANOVA, $n=8$; Figure 6.12a). This was the only group in the study that had group mean ratios on various days which were significantly different from the day 11 ratios. Repeated intra-articular injections of 100 μ M ralfinamide resulted in the mean LWT ratios on days 15, 16 and 17 being statistically higher in comparison to day 11 ($P<0.05$, one-way ANOVA, $n=8$; Figure 6.12b).

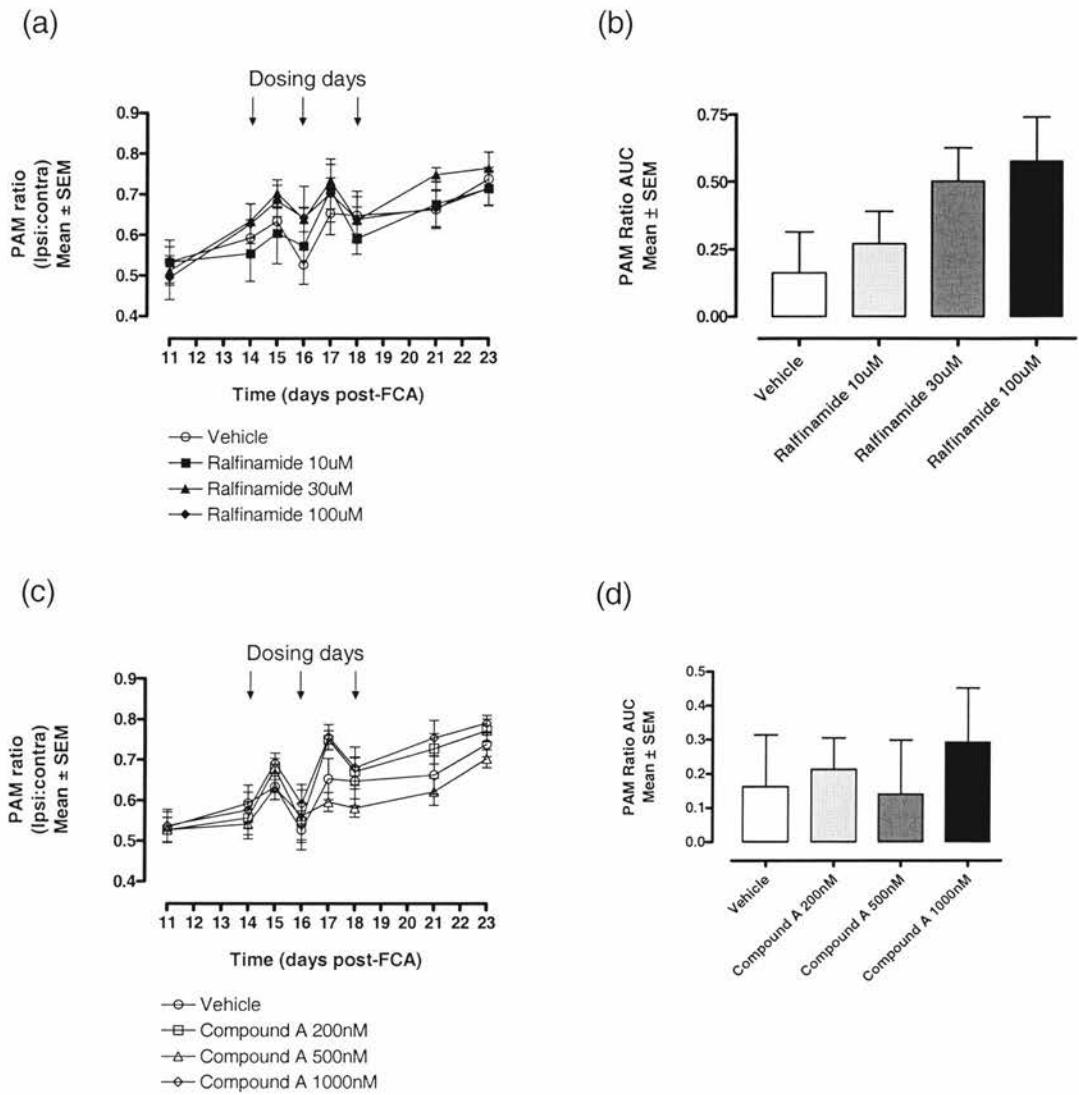


Figure 6.11 Graphs showing the effect of (a) and (b) ralfinamide and (c) and (d) Compound A on the group mean LWT ratio on days 11-21 when the various doses of drug were administered directly into the knee joint on days 14, 16 and 18. There was no significant difference in the group mean LWT ratio for any of the doses of either ralfinamide or Compound A at any time point compared to vehicle ($P>0.05$, two-way ANOVA, $n=8$; Figure 6.11a and 6.11c). There was also no significant difference in the AUC values of days 14, 16 and 18 for the three doses of ralfinamide (b) or Compound A (d) in comparison to vehicle ($P>0.05$, Kruskal Wallis ANOVA, $n=8$).

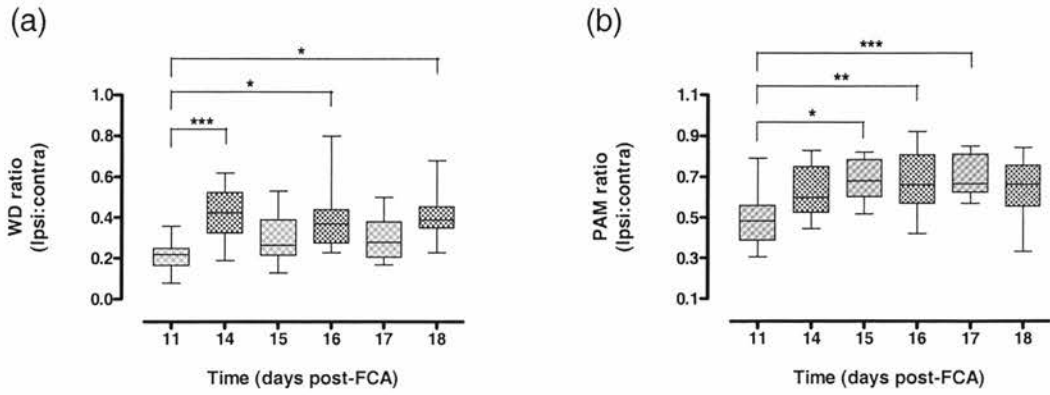


Figure 6.12 Box and whiskers plot summarising the apparent reduction in joint hypersensitivity of repeated intra-articular injections of 100µM ralfinamide in (a) the WD ratio and (b) the LWT ratio. Injections were on days 14, 16 and 18 (signified by the darker symbols) and behavioural assessments were made 1 hour after the injection. (a) The WD ratio on days 14, 16 and 18 were significantly higher than the day 11 WD ratio and (b) the LWT ratio on days 15, 16 and 17 were significantly higher than the day 11 value ($P < 0.05$, one-way ANOVA, $n = 8$).

6.4 Discussion

6.4.1 Discussion of results from IHC studies

Expression levels of the VGSCs $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$ were studied in the cell bodies of neurons which innervated the knee joint during the course of a well established preclinical model of chronic joint pain (Donaldson et al. 1993). FCA injected into the intra-articular space of the knee joint produced a localised unilateral inflammation and established a 'chronic' joint hypersensitivity which was maintained for up to 60 days post-FCA (Wilson et al. 2006). The present study examined expression levels at weekly intervals up to 28 days post-FCA during a period when the joint hypersensitivity was stable but the levels of inflammation were reducing. The fluorescent retrograde tracer FB was successfully used to show the DRG cell bodies of L3-L5 neurones which exclusively innervated the inflamed knee joint (Salo and Theriault 1997). The results showed that there was an overall increase in the expression of all three channels in the knee joint afferent population following injection of FCA. More specifically, on days 14 and 28 post-FCA the percentage of all three channels

in the FB population was significantly greater than that in control animals, with Na_v1.9 also being significantly higher on day 21 post-FCA.

6.4.1.1 Na_v1.7 expression following FCA-induced inflammation

In the present study FCA caused a significant increase in the number of Na_v1.7 positive neurones within the distinct FB population of L3-L5 ipsilateral DRG on days 14 and 28 post-FCA. Previously published reports in rats have shown that intra-plantar injection of carrageenan increased channel mRNA and protein expression in L4/L5 DRG neurones, measured four days after the injection (Tanaka et al. 1998; Black et al. 2004). In a similar model using an intra-plantar injection of FCA, the labelling of Na_v1.7 in L4-S1 DRG neurones 24 hours post-FCA injection was significantly increased (Gould et al. 2004). A study evaluating the expression of this channel in human teeth, removed due to serious pain, revealed a dramatic increase in Na_v1.7 in the nodes of Ranvier and at sites of demyelination in the pulpal tissue, which the authors suggest may contribute to the development of dental pain (Sorensen et al. 2003). The present study provides the first evidence that the upregulation of Na_v1.7 is maintained for at least 28 days following an inflammatory insult. This suggests that Na_v1.7 may not only play an important role in the establishment of inflammatory pain, but could also be crucial for maintaining the chronic nature of the pain even after the inflammation has been resolved (Strickland et al. 2007). The role of Na_v1.7 in experimental inflammatory pain is further strengthened by studies in mice where knocking down or knocking out the channel in nociceptive DRG neurones substantially reduced inflammatory pain behaviours and mechanical and thermal hypersensitivity associated with inflammatory pain (Nassar et al. 2004; Yeomans et al. 2005). All these results corroborate the recent clinical studies describing the importance of Na_v1.7 in the lack of pain sensation in the medical condition CIP where a mutation renders the channel dysfunctional (Cox et al. 2006; Ahmad et al. 2007; Goldberg et al. 2007). Conditions such as paroxysmal extreme

pain disorder (Fertleman et al. 2006) and inherited erythromelalgia (Waxman and Dib-Hajj 2005; Lee et al. 2007) are caused by spot mutations in the channel which lead to a hyperexcitability in the neurones the channels are expressed on and consequently increased nociceptive afferent firing.

6.4.1.2 *Nav1.8 expression following FCA-induced inflammation*

A large body of research exists surrounding the role of Nav1.8 in both the initiation and maintenance of chronic inflammatory pain (Ekberg and Adams 2006). Nav1.8 expression is rapidly upregulated in the early stages of inflammation following either a single injection of FCA or repeated injections of PGE₂, suggesting the channel plays a key role in the initial induction of hypersensitivity (Gould et al. 2004; Villarreal et al. 2005). Support for a role of Nav1.8 in the maintenance of inflammatory pain comes from studies reporting that there is a significant increase in mRNA in DRG tissue four days after carrageenan injection in the food-pad (Tanaka et al. 1998). Moreover, the increase in channel expression is likely to be associated with increased distribution of the channels to peripheral nerve terminals as FCA-induced inflammation results in an increase in the number of Nav1.8 channels observed in peripheral axons (Coggeshall et al. 2004). Studies in Nav1.8 null mutant mice show a delayed hypersensitivity following inflammatory insult (Akopian et al. 1999) and Nav1.8 knockdown mice showed reduced pain behaviours following a peripheral inflammatory insult (Khasar et al. 1998; Joshi et al. 2006). In the present study no significant increase in Nav1.8 was observed at the earliest time point on day 7 post-FCA, but there were significant increases in the expression of Nav1.8 on days 14 and 28 within neurons which innervated the knee joint. This observation is in accord with the previously published studies suggesting a role for the channel in maintaining a chronic hypersensitivity. The channel may therefore play an important role in the immediate initiation in the

hypersensitivity following the FCA, but the time points chosen in this study did not enable us to gain any information about expression levels at 24 and 48 hours post-FCA.

6.4.1.3 *Na_v1.9 expression following FCA-induced inflammation*

Results from the current study show that Na_v1.9 is upregulated in knee joint afferent DRG on days 14, 21 and 28 days post-FCA. Previous studies have also described an upregulation in the mRNA and expression of the channel following FCA administration up to one week after the insult (Tate et al. 1998; Amaya et al. 2006). Na_v1.9 null mutant mice show a reduced hypersensitivity to inflammatory pain induced by FCA, carrageenan, formalin and prostaglandin E₂ (Priest et al. 2005). Interestingly Black et al. (2004) reported no change in the expression of Na_v1.9 mRNA and protein in DRG four days after an intra-plantar carrageenan injection in rats. Furthermore a decrease in the expression of the channel on primary afferent nerves in the periphery 2 days after FCA-induced inflammation has been described, with the authors concluding that Na_v1.9 does not play a role in the peripheral sensitisation that occurs during the early stages of inflammation (Coggeshall et al. 2004). There appears to be conflicting evidence describing the expression of the Na_v1.9 channel in the early stages of inflammatory pain (<day 7). Considering these previous studies and the results presented here, where upregulation is observed in the first instance on day 14 post-FCA (Strickland et al. 2007), it may be concluded that Na_v1.9 does not play a role in the establishment of the initial pain following an inflammatory insult, but does play a crucial role in the maintenance of chronic pain.

6.4.1.4 *Use of FB to highlight the knee joint afferent population*

The use of fluorescent retrograde tracers for tracking peripheral nerves through to their parent cell bodies is a well established technique (Kristensson 1970; Kuypers et al.

1979; Kuypers and Huisman 1984). FB can be transported over long distances, showing pronounced fluorescence in the cytoplasm of parent cells only 48 hours after injection around the nerve terminals (Bentivoglio et al. 1980b). The use of fluorescent dyes *in vivo* to identify knee joint afferent cell bodies in DRG tissue is well described (Hanesch and Heppelmann 1995; Ivanavicius et al. 2004). In this study 82% of the cell bodies that were counted were found in L3 and L4 DRG, a result that concurs with data obtained using the retrograde tracer Fluoro-Gold where 88% were found in L3 and L4 DRG (Salo and Theriault 1997). There was no observable increase in the intensity of the fluorescence in cells of DRGs taken from animals with longer survival times, a trend that has previously been reported (Bentivoglio et al. 1980b). However it must be noted that in the present study the intensity of the fluorescence was not quantified but merely qualitatively assessed by comparing slides at different times after induction of joint hypersensitivity. A phenomenon that was observed in the current study was the fluorescing of glial cells surrounding the retrogradely labelled cell bodies. This has been previously reported as being due to the diffuse migration of dye from the cell bodies into the adjoining satellite cells, and is thought to become more prominent the longer the dye is present in the animal (Bentivoglio et al. 1980a; b). In certain circumstances this might be of concern as it could potentially hinder the correct identification of positive cells within the DRG. However due to the intensely bright fluorescence of the cytoplasm in the present study it can be confidently assured, that only those neurones with retrogradely labelled knee afferents were positively identified.

6.4.1.5 IHC summary

The significant increases in the expression of the VGSCs on days 14, 21 and 28 post-FCA observed in this study correlate with the presence of FCA-induced joint hypersensitivity as measured by a reduction in the WD ratio. A previous study using the same FCA-induced knee joint model of hypersensitivity showed significant increases in the

levels of CGRP within all L3/L4 DRG neurones up to 35 days after intra-articular FCA administration, correlating with a behavioural hypersensitivity of the joint (Staton et al. 2007). It would be interesting to determine whether this increase in CGRP expression takes place solely in the knee joint afferent population, or whether the injection has global effects within the whole DRG population. Similar studies profiling changes in other key channels and receptors, such as the cannabinoids, opioids and the prostaglandins, which are known to play a key role in inflammatory hypersensitivity, may provide further insight into the multiple mechanisms involved in the establishment and maintenance of chronic inflammation-induced pain.

In summary, a significant increase was observed in the channel expression levels of the VGSCs; $\text{Na}_v1.7$, $\text{Na}_v1.8$ and $\text{Na}_v1.9$ in a distinct group of ipsilateral L3-L5 DRG neurones using a model of FCA-induced, chronic joint pain. The sensitisation of primary afferent neurones in acute inflammatory pain has previously been reported to be due to changes in the expression of several VGSC's, although the process clearly involves considerably more than just the modulation of a single class of ion channels. Nevertheless, a retrograde tracing technique was successfully used to demonstrate that $\text{Na}_v1.7$ and $\text{Na}_v1.8$ are upregulated in afferent neurones specifically innervating the site of chronic inflammatory pain. Moreover, this study has increased our understanding of the neurobiology of inflammatory pain by revealing an increased expression in $\text{Na}_v1.9$ channels 14 days post-FCA, highlighting a potential role of this channel in the maintenance of inflammatory hypersensitivity.

6.4.2 Discussion of results from behavioural studies

In this study the effect of ralfinamide or Compound A on FCA-induced joint hypersensitivity was assessed using the WD and LWT behavioural readouts. At first sight it appeared that neither drug at any of the doses used had any analgesic effect on the joint

hypersensitivity. Comparing the responses in the ralfinamide and Compound A groups with that of the vehicle group showed no significant differences in either the WD or LWT ratio on the individual days. Furthermore, there was no significant difference between the drug groups and the vehicle group when the cumulative effect was investigated using the AUC values calculated from the dosing period. The AUC values for the ralfinamide treated animals were not statistically different to the vehicle group, but did appear to be biologically significant with the increasing doses following a trend to cause an increase in both the WD and LWT ratio. Further investigation revealed that with the highest dose of ralfinamide investigated (100 μ M), there was a significant increase in both the WD and LWT ratios on certain days during the dosing period when compared with the day 11 pre-treatment values. This was the only group for either of the drugs to show an increase in the WD or LWT ratio at any time point when compared to the day 11 value. The WD ratio readout for 100 μ M ralfinamide revealed significant increases in the group mean ratio on the dosing days of day 14, 16 and 18 when compared to the group mean ratio on day 11. LWT readouts revealed significant differences between the mean ratios on days 15, 16 and 17 in comparison with day 11 pre-treatment values. These effects were then absent on day 21. These findings demonstrate that the dosing of compounds directly into the knee joint can provide an effective analgesia as measured using two different behavioural readouts. More specifically, the sodium channel blocker ralfinamide at a dose of 100 μ M attenuated the FCA-induced hypersensitivity during the dosing period.

6.4.2.1 Ralfinamide and Compound A

In a previous study employing the same model of FCA-induced knee joint hypersensitivity as described (Wilson et al. 2006), no significant analgesic effect was observed following oral dosing of ralfinamide at 10mg.kg⁻¹ (in house, GlaxoSmithKline). In a rat model of acute inflammatory pain evoked by FCA administered into the footpad (Butler

et al. 1992), both oral and intra-peritoneal ralfinamide at doses of approximately 35 μ M and 105 μ M caused a significant, dose dependent, decrease in allodynia, measured using von Frey filaments (Veneroni et al. 2003). Furthermore, patch clamp studies in rat DRG neurons held at -70mV showed ralfinamide had an IC₅₀ of 55 μ M and 22 μ M at the TTX-resistant and an TTX-sensitive sodium channels respectively although the effects of the drug were more pronounced on the TTX-resistant channels (Stummann et al. 2005). Other studies revealed ralfinamide had an IC₅₀ of 22 μ M in human Na_v1.8 channels held at -80mV (Dekker et al. 2005). Data published after the completion of the current study shows that a dose of 25 μ M in the presence of 0.5 μ M tetrodotoxin suppressed afferent hyperexcitability, with the mean number of action potentials in capsaicin positive nociceptive DRG neurons being reduced from 10.6 \pm 1.8 to 2.6 \pm 0.7 action potentials in every 600ms (Yamane et al. 2007). In view of this evidence, the doses of 10 μ M, 30 μ M and 100 μ M selected for the present study were reasonable, although it is perhaps no surprise that the 10 μ M dose did not cause any effect, and in hindsight a dose of 300 μ M may have provided a better indication of the analgesic properties of the drug.

Relatively little is known about Compound A, with no data published to date. Data generated at GlaxoSmithKline suggests that Compound A is only selective for Na_v1.8 up to 158nM, and then both Na_v1.8 and Na_v1.3 up to 500nM (oral communication, Dr. Paula Green, GSK). The doses of 200nM, 500nM and 1000nM were chosen in order to try and selectively target Na_v1.8 using the lower doses, whilst investigating the compound as a universal sodium channel blocker at the higher dose. The lack of analgesic effect of the drug in any of the doses studied is surprising, as data generated from *in vivo* studies showed that an oral dose of 3mg.kg⁻¹ caused a significant analgesic effect in FCA-induced joint pain. It could be that metabolites produced when the drug is given orally are responsible for its analgesic action, and administration into the knee joint resulted in a different fate of the drug, where these metabolites were not produced.

A consideration to take into account is the fact that Compound A is highly insoluble in either distilled water or saline. In order to get the compound into solution a vehicle containing 2% DMSO was used. *In vitro* studies have shown that DMSO at 1-5% can alter nerve conduction velocity and action potential propagation in ventricular tissue (Ogura et al. 1995), and topical application of 50% DMSO provides analgesia in human rheumatoid arthritis (Abdullaeva and Shakimova 1989). The presence of 2% DMSO in the vehicle during this study may account for the positive AUC value observed in the vehicle dosing group (if the vehicle had no effect then the AUC value would be zero). This vehicle effect makes it very difficult to achieve a statistically significant difference between the test drugs and vehicle as described above. This being considered, the vehicle was prepared with the lowest possible amount of organic solvent necessary in order to solubilise Compound A and was administered directly into the knee joint, reducing the chances of observing any centrally mediated side effects.

6.4.2.2 Differences in results obtained from the LWT and WD readouts

For both, WD and LWT, displaying the results as a ratio provided a clear method of presenting the data as in both cases the un-injected limb acts as an internal control. In this way any random day to day changes can be accounted for. Interestingly the analgesic effect of 100 μ M ralfinamide as measured using the WD ratio was only obtained on the dosing days, whereas the analgesic effects measured using the LWT ratio were seen the day after the dosing. This may be because injection into the knee joint caused superficial acute pain, as a result of tissue damage. As the PAM LWT readout requires the knee joint to be squeezed, the pain associated with the injection 1 hour previously may result in a falsely decreased LWT, masking the analgesic effect of the ralfinamide on the underlying FCA-induced chronic pain of the joint. In future studies the insertion of a needle into the un-injected limb

could act as an internal control for this potential acute pain associated with the dosing procedure.

6.4.2.3 Intra-articular administration of drugs

In this study, test drugs were administered directly into the FCA-injected knee joint. This route is clinically relevant as drugs are often given into the knee joint of patients suffering from OA or RA (Christophidis and Huskisson 1982; Stein et al. 1991; Stein et al. 1996a). Repeated intra-articular doses are not regularly used in the clinic due to the risk of infection at the injection site. In the present study, on rare occasions leakage of a small amount of drug solution was observed from the injection site. This was probably due to the joint space in FCA-injected animals being slightly less than the estimated 100 μ l (Barton 2007). It is possible that a residual amount of drug was retained in the joint following the first dose that could affect the overall concentration of drug in the knee when the subsequent doses were given. This could account for the unexpected differences in LWT ratio of the 100 μ M ralfinamide animals on the non-dosing days 15 and 17 compared to day 11.

Dosing directly into the knee joint required the animals to be briefly anaesthetised. Taking into account the initial injection of FCA animals were anaesthetised four times and as mentioned in the results above two animals died whilst under anaesthesia. The contribution of the halothane anaesthetic towards any analgesic effect needs to be considered as in vitro studies have demonstrated halothane can block TTX-resistant channels in the rat DRG causing a shift in inactivation threshold (Scholz et al. 1998). However at the low concentrations used during the temporary anaesthesia it is unlikely that halothane will contribute to any decrease in joint hypersensitivity, especially as the behavioural tests took place 1 hour after anaesthesia.

6.4.2.4 Behavioural study summary

The results of this study demonstrate analgesia in a model of chronic inflammatory joint pain can be achieved using the sodium channel blocker ralfinamide when a dose of 100 μ M is injected directly into the FCA-injected knee. Ralfinamide, which is thought to predominantly act by blocking the TTX-resistant sodium channels Na_v1.8 and Na_v1.9, caused a significant attenuation of the FCA-induced reduction in the WD and LWT ratios. This suggests that there is afferent nerve discharge that is mediated, in part, by VGSCs present in the peripheral nerves innervating the knee joint. An obvious next step would be to investigate whether higher doses of ralfinamide would cause a more significant attenuation of the FCA-induced joint hypersensitivity. Although rotarod studies from previously published data suggested that the doses of ralfinamide and Compound A investigated would not cause motor impairment, it would be wise to establish this in future studies, as injection directly into the knee joint may have a more pronounced effect than systemic administration of the drugs. The results obtained from the three doses of Compound A showed no significant effect on either the WD or LWT ratios, but this can be attributed to the low doses of drug chosen to achieve selective blockade of Na_v1.8. Further studies with higher doses of this compound may reveal an analgesic effect of the compound that was missed in this study due to the low concentrations injected into the knee joint.

6.4.3 Sodium channel chapter summary

The results from the studies described in this chapter add to the wealth of literature describing the roles, modulation and regulation of the VGSCs Na_v1.7, Na_v1.8 and Na_v1.9 in chronic pain associated with an inflammatory insult (for reviews see Amir et al. 2006; Cummins et al. 2007). The increase in the expression of the channels in the rat knee joint afferent cell body population in DRGs on days 14, 21 and 28 suggests that each channel may play an independent role in the maintenance of inflammatory hypersensitivity in the long

term by increasing neuronal hypersensitivity and spontaneous discharge. The sodium channel blocker ralfinamide successfully attenuated the FCA-induced joint hypersensitivity on day 14-18 post-FCA, a time point at which these channels were shown to be upregulated. Sodium channels remain a promising and potentially viable target in the development of novel peripherally acting analgesics for the treatment of chronic inflammatory pain. Given the potentially lucrative market for novel pain therapies, drugs that are able to selectively block these channels may provide analgesia with limited side effects. It remains to be seen whether the recently synthesised $\text{Na}_v1.8$ selective blockers MrVIB (Bulaj et al. 2006; Ekberg et al. 2006) and A-803467 (Jarvis et al. 2007) will be effective in providing successful patient analgesia.

Chapter 7
General discussion

7.1 Development of PAM

7.1.1 Major findings

- The PAM device has been developed and validated as a novel behavioural tool for assessing localised mechanical hypersensitivity of rodent knee joints.
- Results show that PAM can be used to obtain quantitative measurements of mechanical hypersensitivity in the knee joint of rats and mice.
- PAM detected the expected analgesic actions of prednisolone, morphine and celecoxib.
- The LWT readouts obtained using the PAM device strongly correlated with results obtained from the established weight distribution readout.
- In summary PAM provides a novel preclinical readout which is similar to assessments made in the clinic using a dolorimeter (Langley et al. 1983).

7.1.2 Limitations of the PAM device

The use of PAM requires the animals to be repeatedly handled and have their hypersensitive knees squeezed. In the present studies all efforts were made to reduce the amount of stress caused to the animals by handling the animals regularly, habituating them to the device and by making the assessments at the same time each day. Despite habituation and conditioning, the practical use of PAM to elicit a limb withdrawal may be a stressful experience for the animals, and analgesia is a well known consequence of activation of the HPA axis in response to stress (Takahashi 1991). The present studies utilised the contralateral limb as an internal control, as well as dosing with vehicle in order to prevent false positive conclusion being drawn as a result of a stress-induced response.

A second limitation of the PAM device is that two operators are needed to make measurements as one person holds the animal while a second operates the device. This is

something that is unusual with regards to behavioural testing in pain studies as the incapacitance tester, analgesymeter, Hargreaves thermal assessment and von Frey filaments all require one operator only, and may therefore be considered a negative feature of the PAM device.

7.1.3 Further investigations with PAM

PAM was designed so that it could be applied to the investigation of hypersensitivity in different joints such as the ankle, or to areas of the body such as the paw which is classically investigated using the Randall and Selitto analgesymeter (Randall and Selitto 1957), or von Frey filaments (von Frey 1894a; b; 1922). The device could now be used as an assessment in preclinical drug discovery studies of inflammatory joint pain. In that respect, PAM has the potential to facilitate the discovery and progression of novel analgesics into the public domain.

A study in which telemetry devices which are capable of monitoring blood pressure in the conscious animal (Kramer and Kinter 2003) were surgically implanted prior to behavioural testing would show if the operation of PAM is stressful for the animals, even after a habituation period. An increase in blood pressure is a general indication of sympathetic nerve activity which can lead to activation of the HPA axis and result in a stress response.

7.2 Opioid receptors on peripheral nociceptors

7.2.1 Major findings

- Results obtained from the behavioural experiments provide clear evidence that functional opioid receptors are present on nociceptive afferents innervating the rat knee joint at 14-18 days post-FCA.

- EM1 or morphine administered directly into the FCA-injected joint, under transient anaesthesia, resulted in a reduction in the knee joint hypersensitivity as measured in the conscious animal.
- Furthermore, this reduction in hypersensitivity was evident in the behavioural assessment performed half an hour after administration of EM1 or morphine, and was still significantly reduced compared to vehicle, four days after dosing.
- Nociceptive discharge from the MAN, evoked by noxious mechanical von Frey filaments, was attenuated by EM1 or morphine in terminally anaesthetised rats during the 'chronic' phase of the joint hypersensitivity.
- This decrease in neural discharge was confirmed as being opioid-mediated, as the opioid receptor antagonist naloxone blocked this attenuation of discharge.
- EM1 caused no change in nociceptive discharge evoked by the von Frey filaments in un-inflamed naïve animals, suggesting the action of EM1 is dependent on an inflammatory induced change in the nociceptors.

7.2.2 Relationship to current understanding

Opioid receptors are present on predominantly small, unmyelinated, primary afferents in naïve rats (Fields et al. 1980; Coggeshall et al. 1997). Expression of μ -opioid receptors increased significantly in the ipsilateral DRG of rats 1-3 days after carrageenan-induced hind-paw inflammation (Ji et al. 1995), yet no increase was seen over a similar time period following FCA-induced hind-paw inflammation (Schafer et al. 1995). Inflammation induced by intra-plantar FCA, enhanced axonal transport of opioid receptors to the periphery, with an accumulation of the receptors observed in homogenized paw tissue (Hassan et al. 1993; Zhang et al. 1998). The increase in opioid receptors observed at the site of inflammation has been complemented by studies performed in rats, in which inflammatory cells containing endogenous opioid peptides infiltrate the site of inflammation

(Stein et al. 1990b). Studies in both mice and rats have focused on the acute phase (0-4 days) of the inflammatory reaction, with strong evidence existing that highlights a peripheral nociceptive action of opioids (Stein 1991).

A study investigating the more long term effects of systemically administered FCA, used immunohistochemical labelling to report an increase in the expression of the μ -opioid receptors in DRG cell bodies at four weeks post-FCA, but not the dorsal horn leading the authors to conclude that the receptors were being transported to the periphery (Ballet et al. 2003). In contradiction to this Li et al., (2005) reported a down regulation in μ -opioid receptor protein and gene expression one week after a monoarthritis was induced in the knee joint using FCA. The results from my studies provide strong evidence that there are functional opioid receptors associated with knee joint afferents during the chronic stage of joint inflammation. This was demonstrated in studies where two different opioid agonists were administered locally, and both were antinociceptive in the behavioural and electrophysiological studies. Moreover, this antinociceptive effect was abolished by the opioid receptor antagonist naloxone, proving that the effect was opioid-mediated.

7.2.3 Clinical implications

Preclinical models of human disease are used by the pharmaceutical industry as part of the drug discovery process to facilitate the finding of novel pharmacological compounds for use in the treatment of the symptoms associated with arthritis. Morphine administered directly into the knee joint in RA sufferers reduces chronic joint pain associated with the inflammatory disease (Stein et al. 1999); EM1 or morphine, administered by the same route, enabled rats to place more weight through their inflamed knee joint, a behavioural indication that locally administered opioids provide analgesia in rats. These results build confidence in the use of FCA-induced inflammatory joint pain as a preclinical model of inflammatory joint pain because the data is comparable to that seen in the clinic.

Chronic joint pain is often treated orally using a combination of two low dose analgesics with different mechanisms of action, for example Ultracet containing the NSAID paracetamol and the opioid tramadol (Emkey et al. 2004). This combination therapy allows the level of pain relief to be maintained whilst minimising the side effects associated with the higher doses of the individual drugs (Emkey et al. 2004; Miranda et al. 2007). It is possible that EM1 could be utilised in such a combination as results presented in this thesis show that it is capable of attenuating the nociceptive signalling from knee joint afferents. Topical application of the opioid lidocaine-prilocaine cream has been shown to be more effective than i.v. morphine in treating post-operative pain associated with thoracostomy tube removal (Valenzuela and Rosen 1999). EM1 in a cream formulation, applied topically in the same fashion as capsaicin cream (Deal et al. 1991), could also be explored as a convenient palliative treatment for chronic joint pain. Experiments would have to be performed to confirm that the drug is absorbed through the skin and into the joint capsule directly, rather than via absorption into the blood stream of the highly vascularised joint, and subsequent circulation to the joint capsule.

7.2.4 Limitations of the data

Despite several attempts with various different, type-specific, opioid receptor antibodies, I was unable to establish an IHC protocol capable of generating fluorescent staining with a clear signal to background ratio, when observing DRG sections under the fluorescent microscope. This meant opioid receptor expression in the knee joint-specific afferent cell bodies could not be investigated. The inclusion of opioid expression data would have been invaluable in providing further evidence as to the changes in opioid receptors in specific DRG during the course of the FCA-induced inflammation. An up-regulation would imply that, increased amounts of opioid receptor are being transported into the periphery.

This finding would, in part, help explain why nociceptive discharges are attenuated by EM1 or morphine in inflamed joint afferents, but not naïve joints.

In the present studies the animals had to be anaesthetised, either terminally for electrophysiology, or transiently when administering the opioids into the knee joint for the behavioural work. It may be that pentobarbital used in the *in vivo* electrophysiology studies modulated the opioid receptors and it would have been useful to conduct repeat experiments using different anaesthetics such as urethane. In order to adhere to Home Office 3Rs, and to remain within temporal and economic restraints of the experimental work, these experiments were not performed. Pentobarbital was chosen as it has a short half life ensuring the level of anaesthesia was more accurately controlled. Furthermore, conclusions drawn regarding the effect of EM1 and morphine were made after integrating the outcome of the electrophysiological experiments and the behavioural studies. Every attempt was made to ensure that these comparisons were fair by ensuring the same weight and strain of rats were used and the time points at which tests were made were kept the same. However, different anaesthetics were used for terminal and transient anaesthesia and the behavioural end-point of weight distribution is a different stimulus to mechanically-evoked discharge. In the electrophysiological experiments the C-fibres were the only type to be identified by conduction velocities. Fibres that were not capsaicin sensitive but responded to noxious mechanical stimulation were assumed to be A δ -fibres, but without a conduction velocity this could not be confirmed.

7.2.5 Further studies

Ideally the expression of the μ , δ and κ opioid receptors would be quantified in knee joint afferent DRG. Extensive but unsuccessful efforts were made at the time to perfect a fluorescent staining protocol, and this challenge could be re-visited to provide valuable data on receptor expression.

The ability of the endogenous opioid peptide EM2 to reduce hyperalgesia in the inflamed knee joint requires investigation, as studies were not performed due to time and cost constraints during the project. Furthermore, the role of opioids in more advanced stages of chronic joint hypersensitivity should be studied in alternative models of inflammation, such as the antigen-induced arthritis model of OA (Buchner et al. 1995).

7.3 VGSCs

7.3.1 Major findings

- The present studies clearly demonstrated that expression of Na_v1.7, Na_v1.8 and Na_v1.9 in the FB labelled DRG population, were upregulated at various times during the 28 day study (see Summary diagram 7.1).
- The fluorescent retrograde tracer FB was successfully used to highlight a population of afferent cell bodies within L3-5 DRG which innervated the knee joint.
- Results from behavioural studies revealed that intra-articular administration of the sodium channel blocker ralfinamide (100µM) produced significant reductions in joint hypersensitivity on the days it was dosed compared to pre-dose values, assessed using both the incapitance tester and the PAM device.

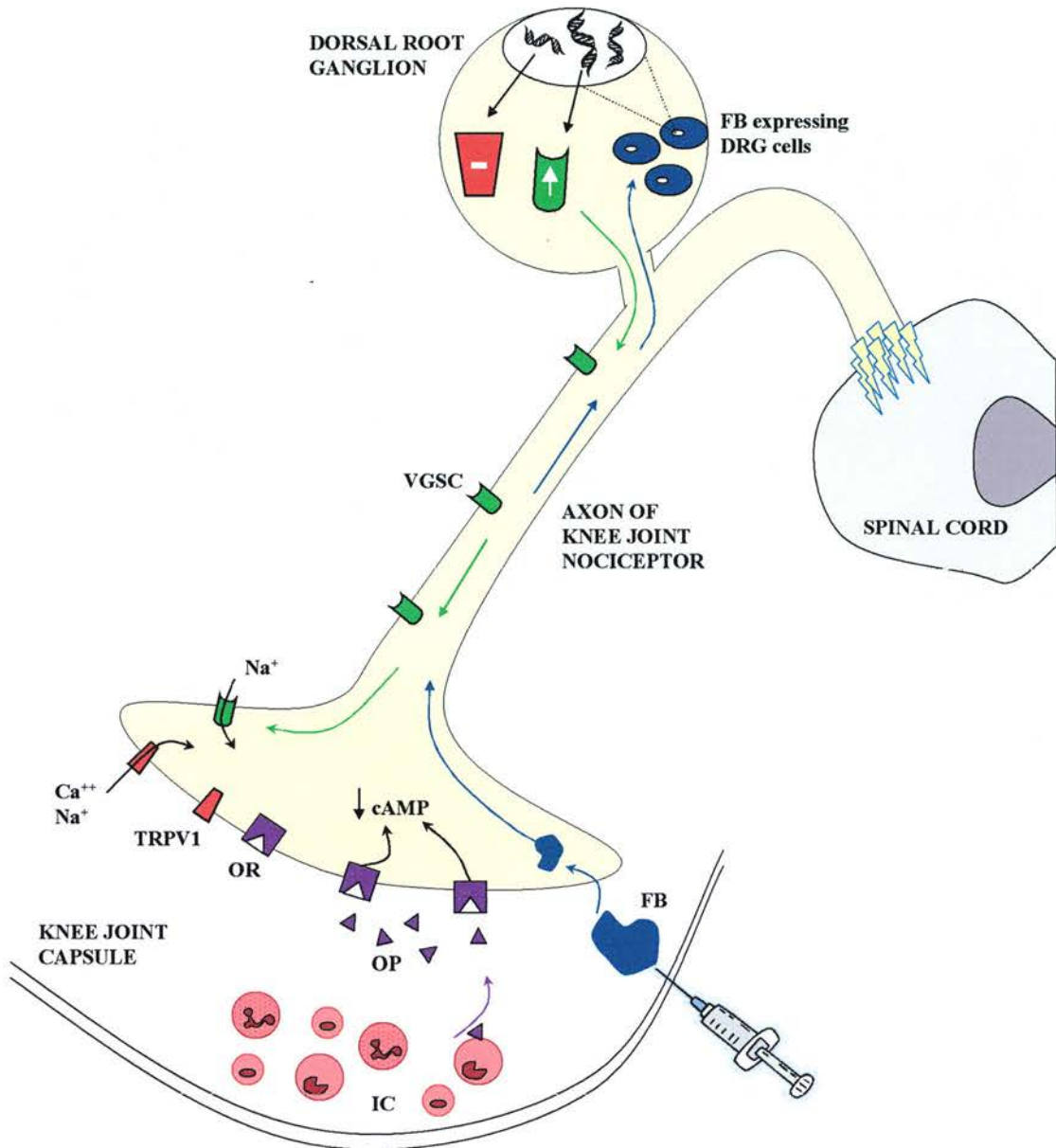


Figure 7.1 Summary of how VGSC expression, opioid receptor signalling, and TRPV1 receptors contribute to nociceptive discharge in nociceptive neurons following FCA-induced joint inflammation. FB was injected into the knee joint and was retrogradely transported to DRG cell bodies innervating the knee joint. Expression of $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$ were increased in FCA-injected joints, whilst TRPV1 expression remained the same. Opioid peptides such as EM1 and morphine were administered into the knee joint where they activated opioid receptors expressed on peripheral terminals. Inflammatory cells have also been implicated in providing analgesia at site of tissue injury by releasing endogenous opioid peptides such as EM1. Coupling of the receptor to the inhibitory G-protein resulted in a decrease in cyclic AMP, leading to a suppression of Na^+ and Ca^{2+} currents, which in turn resulted in a decreased neuronal hyperexcitability. FB = fast blue, \uparrow = increase in VGSC expression, - = no change in TRPV1 expression, OP = opioid peptides, OR = opioid receptors and IC = inflammatory cells

7.3.2 Relationship to current understanding

The present studies are in keeping with published papers reporting an increase in expression of the $\text{Na}_v1.7$, $\text{Na}_v1.8$ and $\text{Na}_v1.9$ channels in rat DRG tissue following inflammatory insults (Black et al. 2004; Gould et al. 2004; Priest et al. 2005; Villarreal et al. 2005). The present results are the first to provide detail concerning changes in specific channels during a rat model of FCA-induced chronic inflammatory joint pain up to 28 days post-FCA (see chapter 6). Gould et al., (1998) investigated the changes in the expression level of all the sodium channels in DRG tissue up to six months after FCA-induced inflammation of the paw. Specific antibodies were used in present studies to investigate individual sodium channel subtypes in knee joint specific cell bodies.

Knockdown of $\text{Na}_v1.7$ in murine DRG decreased both acute and chronic pain behaviours (Nassar et al. 2004; Yeomans et al. 2005), and intraplantar FCA resulted in an increase in the levels of channel labelling in DRG 24 hours after the insult (Gould et al. 2004). It is therefore not surprising to find that $\text{Na}_v1.7$ was up-regulated on days 14 and 28 post-FCA in the present study. Expression levels of $\text{Na}_v1.8$ and $\text{Na}_v1.9$ were also increased on day 14-18 post-FCA. This is also in keeping with studies in rats where these subtypes were upregulated in DRG following inflammatory insults in acute studies (Tanaka et al. 1998; Tate et al. 1998; Black et al. 2004; Coggeshall et al. 2004). The present results are the first to demonstrate a role for these sodium channels in the maintenance of chronic inflammatory pain after the inflammation has resolved. An increase in sodium channel expression in nociceptive afferents will cause neural hyperexcitability and lead to an increase in the spontaneous discharge that is associated with chronic pain.

7.4 General conclusions

The main finding of this thesis is that functional opioid receptors are present in joint afferents innervating the knee joint 14-23 days after inducing joint inflammation with FCA.

In behavioural studies, morphine or EM1 administered directly into the knee significantly attenuated the FCA-induced joint hypersensitivity. In addition, *in vivo* electrophysiology recordings taken from the MAN showed that both morphine and EM1 reduced neural discharge evoked by von Frey filaments in FCA-injected knee joints. The attenuation of nociceptive discharge was confirmed as being mediated through opioid receptors by demonstrating that this reduction in action potential discharge was antagonised by naloxone. The conclusions reached from the opioid work presented in this thesis suggest that controlling the release of endogenous opioids at the sites of inflamed tissues, or restricting exogenous opioids to the periphery, may provide excellent long term analgesia in chronic inflammatory pain, whilst avoiding the common centrally mediated side effects.

A further finding is that the VGSC subtypes: Na_v1.7, Na_v1.8 and Na_v1.9 contribute, in part, to maintaining chronic neural hyperexcitability following an inflammatory insult. All three channels were upregulated in the cell bodies of afferents innervating the knee joint 28 days after the initial inflammatory insult (see Figure 7.1). Behavioural tests showed that there was a significant joint hypersensitivity on day 28 post-FCA, and *in vivo* electrophysiology experiments showed that spontaneous neural discharge was increased in FCA-injected animals compared to naïve animals. These observations could be due, in part, to the increased expression of the Na_v1.7, Na_v1.8 and Na_v1.9 on knee joint afferents in FCA-injected animals on days 14 and 28 post-FCA. Investigations into whether ralfinamide reduces this spontaneous activity observed in the *in vivo* electrophysiological recordings, would confirm this role.

Additionally, IHC studies showed no difference in the levels of TRPV1 receptor expression in knee joint afferent DRG cell bodies between naïve and FCA-injected animals. This data complements the *in vivo* electrophysiology finding that capsaicin-evoked neural firing was unchanged in FCA-injected animals.

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Appendix I
Associated publications

A Pharmacological comparison of two different sensory readouts in a rat model of chronic inflammatory joint pain

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The aim of this study was to compare two different methods of evaluating the efficacy of known analgesics, currently available in the clinic, for the treatment of the chronic pain associated with rheumatoid arthritis. The paw pressure withdrawal threshold assay (Randall & Selitto, 1957) has long been used as a mechanical test of assessing hypersensitivity in models of acute inflammatory pain (Anseloni *et al.*, 2003). The recent development of the weight bearing assay has seen the introduction of an alternative readout providing an objective assessment of incident hypersensitivity (Clayton *et al.*, 1997). Using a rat model of chronic inflammatory joint pain (Donaldson *et al.*, 1993), a monoarthritis was induced by injecting 150µl (1mg/ml) of Freund's complete adjuvant (FCA) into the intra-articular space of the knee joint. Injections were done under brief anaesthesia (3% isoflurane with O₂ at 1.5l.min⁻¹). A series of six, blind and randomised experiments (n=40, for each experiment) were conducted assessing the analgesic potency of Rofecoxib (1, 3 and 10mg.kg⁻¹), Etoricoxib (1, 3 and 10mg.kg⁻¹) and Ibuprofen (3, 10 and 30mg.kg⁻¹), using both the paw pressure withdrawal threshold and the weight bearing readouts. Drug or vehicle (DMSO 1%, distilled water 33% and PEG₄₀₀ 66%) were chronically dosed (p.o.) on days 13-17 post FCA injection and the above readouts were used to assess the ability of the test drug to reverse the induced hypersensitivity. The contralateral paw pressure withdrawal threshold readout can be taken to negate any compound-induced motor deficits being falsely concluded as analgesia. All data were transformed into an area under the curve (AUC) value, for days 13-17 for each dosing group. Using Statistica V.6 the AUC data were statistically analysed by an ANOVA followed by a Duncan's post-hoc test. A difference in groups was only considered significant when P<0.05. The relationship between the AUC group means were then assessed using Pearson's linear regression correlation analysis in order to produce a correlation coefficient (where 1 = positive correlation, 0 = no correlation and -1 = negative correlation) and an associated P value stating the statistical significance of the test. Results revealed strong correlations between the two readouts (Rofecoxib r = -0.9949, Etoricoxib r = -0.9834 and Ibuprofen r = -0.9514) with both assays producing similar 50% effective dose (ED₅₀ values) for each drug. These results provide evidence that the weight bearing assay can be used alongside the paw pressure withdrawal threshold assay to increase confidence in the pre-clinical potency of novel analgesics and hopefully lead to improved clinical efficacy.

Clayton, NM; Oakley, I; Thompson, S; Wheeldon, A; Sargent, B; Bountra, C. (1997) *BJP*, **120**, 219P.

Donaldson, LF; Seckl, JR & McQueen DS. (1993) *JNM* **49**, 5-10.

Randall, LO; Selitto, JJ. (1957) *AIPT*. **111**, 409-419.

A novel behavioural readout for assessing hypersensitivity of knee joints in a murine model of unilateral arthritis

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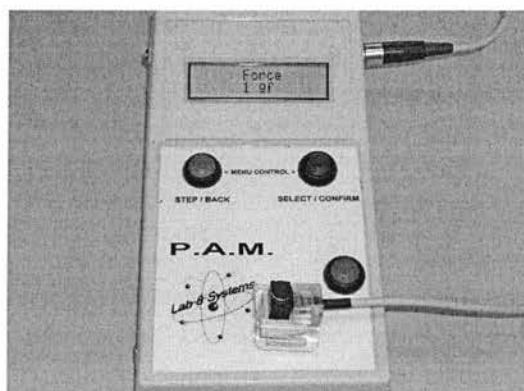
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The aim of this study was to develop a new quantitative method for measuring hypersensitivity of the knee (stifle) joint during a murine model of unilateral arthritis (Gauldie *et al.*, 2004). The pressure application measurement device (PAM) is designed to objectively assess a discrete localised hypersensitivity across the knee joint. The force applied across a joint under test is measured by a force transducer fitted to the experimenter's thumb (see Figure 1). A gradually increasing 'squeeze' force was applied across the joint and by means of calibrated instrumentation the force in grams applied was recorded. The test endpoint was when the animal withdrew its limb. Male C57BL6 mice (n=24, 23-27g) were transiently anaesthetised (3% isoflurane with O₂ at 1.5l.min⁻¹) and a small incision was made over the knee joint to show the patella tendon, sham (n=8) animals recovered with no further treatment. The remaining 16 animals were injected with Freund's complete adjuvant (FCA; 20µl, 10mg.ml⁻¹) under the patella tendon into the intra-articular space of the joint. Prior to surgery and for 21 days following PAM withdrawal threshold, weight bearing (Clayton *et al.*, 1997), knee diameter and body weight were recorded. On day 10 the FCA injected animals were ranked and randomised into two matched groups (n=8) and dosed (s.c.) on days 13-17 with either prednisolone (1mg.kg⁻¹) or vehicle (ethanol 5%, PEG 45% and distilled water 50%). PAM data were transformed into an area under the curve (AUC) value and analysed using a Mann-Whitney test, where p<0.05 was considered significant. Results showed FCA injected animals had significantly lower withdrawal thresholds than sham on day 3 (p= 0.0001), when compared to day 0, confirming the FCA induced hypersensitivity. Over days 13-17 the prednisolone treated group had significantly higher AUC values than the vehicle group (p= 0.0104), compared to day 10, due to a reversal of hypersensitivity. PAM results correlated significantly with weight bearing data (r= 0.9, Pearson's correlation). These results show that PAM is a robust tool which provides an easy to use objective method for localised assessment of knee joint hypersensitivity in mice, which correlates strongly with a previously accepted behavioural readout.

Clayton *et al.*, 1997 *Br J Pharmacol* **120**:219P.

Gauldie *et al.*, 2004 *J Neurosci Methods* **139**(2):281-291.

Figure 1. Pressure application measurement device (PAM)



Intra-articular morphine is antinociceptive in a rat model of chronic joint inflammation

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Intra-articular injection of morphine produces a long lasting analgesia in both rheumatoid and osteoarthritis (Likar *et al.*, 1997; Stein *et al.*, 1999). Recent electrophysiological data has provided evidence inconsistent with these clinical observations, showing a loss of antinociception to the μ -opioid agonist endomorphin-1, in 7 day arthritic animals (Li *et al.*, 2005). The aim of this *in vivo* electrophysiological study was to investigate the antinociception of morphine, in male Wistar rats (200-300g) induced with arthritis by Freund's complete adjuvant (FCA) injected 14-28 days prior to the study (Wilson *et al.*, 2005), using the TRPV1 agonist capsaicin as an algogenic stimulus. Rats were anaesthetised using pentobarbital (60mg/ml, 0.1ml/100g i.p. maintained by i.v. infusion of 7.8mg/hr) and neuronal recordings were taken from the medial articular afferents of the left knee joint (Gauldie *et al.*, 2001). Capsaicin (1 μ g) was given every 45 mins as the test protocol. Morphine (300 μ g) or saline was then given after the second control application of capsaicin. Drugs were injected close to the knee via an intra-articular femoral catheter. A Wilcoxon signed rank test was used to assess the statistical significance in any change in firing frequency evoked by capsaicin before and after, either saline, or morphine. A Mann Whitney test was used to show there was no significant difference in the firing frequency at time point 0 between the saline group (A.) and the morphine group (B.). Null hypothesis was rejected at $P < 0.05$.

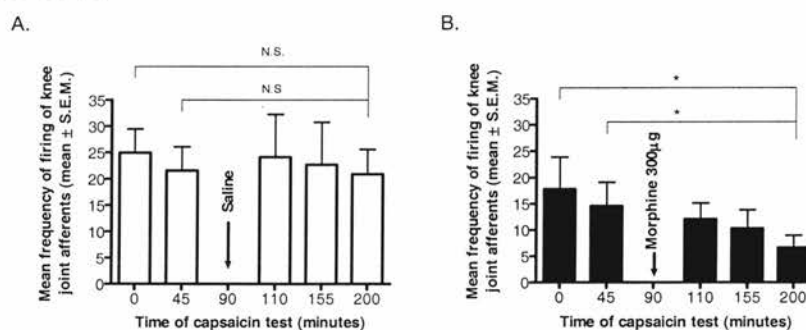


Figure 1. The mean firing frequency of knee joint afferents (spikes/second) in response to repeated capsaicin (1 μ g) doses. Control experiment (A. $n=6$) shows no significant change in the capsaicin evoked firing. Morphine (300 μ g) experiment (B. $n=6$) shows a significant change in the firing frequency, 110 minutes after the morphine when compared back to both controls.

The change in firing frequency from the 45 minute control application of capsaicin compared to the final application was from 21.54spikes/sec to 20.86spikes/sec with saline, a 3% decrease, and from 14.55spikes/sec to 6.64spikes/sec with morphine, a 54% decrease. Morphine significantly reduced nociceptive discharge evoked by capsaicin in arthritic animals. This is in accord with the clinical data that intra-articular morphine is a potent analgesic in inflammatory arthritis. Our results suggest that μ -opioid receptors are functional in the later stages (days 14-28) of this model of arthritis. Further studies using the selective μ -opioid agonist endomorphin-1 and the opioid antagonist naltrexone are needed to help characterise the antinociceptive action of morphine and the receptors involved.

Gauldie, S.D. *et al.* (2001) *Br J Pharmacol* **132**, 617-621

Li, Z. *et al.* (2005) *Arthritis Rheum* **56**, 3210-3219

Likar, R. *et al.* (1997) *Anesth Analg* **84** 1313-1317

Stein, A. *et al.* (1999) *Pain* **83** 525-532

Wilson, A. *et al.* (2005) *Eur J Pain* in press

Aim

The aim of this study was to investigate the peripheral antinociceptive effect of morphine on capsaicin induced firing of the medial articular nerve (MAN), using electrophysiological techniques in a rodent model of chronic joint inflammation (Wilson *et al.*, 2005).

Introduction

Intra-articular injection of morphine produces a long lasting analgesia in both rheumatoid- and osteo-arthritic human patients (Likar *et al.*, 1997; Stein *et al.*, 1999). Recent electrophysiological data from studies in rats has provided evidence inconsistent with these clinical observations, showing a loss of antinociception to the μ -opioid agonist endomorphin-1, in 7 day arthritic animals (Li *et al.*, 2005).

Methods

Experiments were performed on 12 adult male wistar rats weighing 200-300g (Charles River, UK). Experiments were performed in accordance with Home Office regulations and within UK animal welfare guidelines, and received Local Ethics Committee approval. A chronic unilateral inflammation of the left knee joint was achieved by an intra-articular injection of 150 μ l of Freund's complete Adjuvant (FCA, 1mg.ml⁻¹ heat killed *Mycobacterium Tuberculosis* suspended in mineral oil, Sigma). Animals were briefly anaesthetised using 3% isoflurane (O₂ at 1.5l.min⁻¹), and had the left knee shaved and cleaned. FCA was injected through the patella tendon into the joint space of the left knee. The animals were allowed to recover from anaesthesia before being returned to cages layered with paper bedding for comfort.

Following the induction of chronic unilateral inflammation (14-28 days post injection) rats were anaesthetised using pentobarbital (60mg/ml, 0.1ml/100g i.p. maintained by i.v. infusion of 7.8mg/hr) and neuronal recordings were obtained from the medial articular nerves of the left knee joint (Gauldie *et al.*, 2001). Drugs were injected in 0.1ml saline close to the knee via a femoral catheter (i.a.) Capsaicin (1 μ g i.a.) was injected every 45 minutes as the test protocol. Morphine (300 μ g) or saline was injected i.a. after the second control application of capsaicin. A Wilcoxon signed rank test was used to assess the statistical significance in any change in firing frequency evoked by capsaicin before and after, either saline, or morphine. The null hypothesis was rejected at $P < 0.05$.

Results

- Morphine significantly reduced the frequency of firing of the medial articular nerves in response to capsaicin, compared to the initial control ($P=0.001$), attenuating the responses by 54% from 14.6 spikes/sec to 6.6 spikes/sec, 110 minutes post- drug administration (see Figure 1) .
- This is in comparison to saline-injected animals, where the frequency of firing of the medial articular nerves in response to capsaicin fell 3% from 21.5 spikes/sec to 20.9 spikes/sec (see Figure 1).
- There was no significant difference, at 0 minutes, in the firing frequency between the saline group (A) and the morphine group (B) (Mann-Whitney)

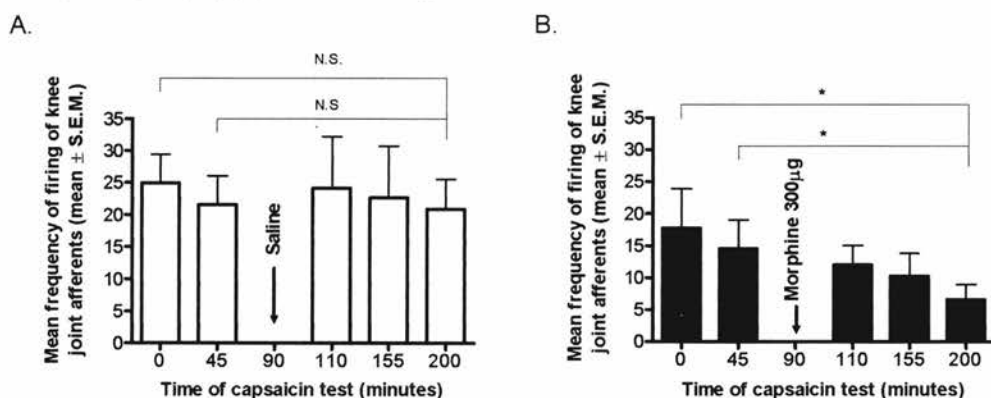


Figure 1. The mean firing frequency (spikes/second) of knee joint nociceptive afferents in response to repeated doses of capsaicin (1 μ g). Control experiment (A. n=6) showed no significant change in the capsaicin-evoked firing. Morphine (300 μ g) experiments (B. n=6) showed a significant change in the firing frequency, 110 minutes after the morphine, in comparison with controls.

Summary and Conclusions

- These results demonstrate that intra-articular morphine is a potent analgesic in this rodent model of inflammatory arthritis.
- Our results indicate that opioid receptors are functional in advanced stages (days 14-28) of the model.
- Further studies using the selective μ -opioid agonist endomorphin-1 and the opioid antagonist naltrexone are needed to characterise the peripheral antinociceptive action of morphine and the receptors involved.

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Likar, R. *et al.* (1997) *Anesth Analg* **84** 1313-1317, Stein, A. *et al.* (1999) *Pain* **83** 525-532,
Wilson, A. *et al.* (2005) *Eur J Pain* in press

Pressure application device (PAD) - A novel behavioural readout for assessing a localised hypersensitivity of the knee joint in a murine model of unilateral arthritis

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Chronic joint pain has high incidence levels in the UK, but with current treatments providing poor pain relief and commonly associated with undesirable side effects, there still remains a great demand for the development of new, safer analgesics. The aim of this study was to assess the novel Pressure application device (PAD) in a mouse model of unilateral hypersensitivity, which mimics mild arthritis in man. PAD is the first mechanical tool designed to assess objectively a discrete localised hypersensitivity directly across the knee joint. We aimed to validate the device in order to improve the translation of novel analgesics from *in vivo* models to man. A gradually increasing 'squeeze' force is applied across the joint and by means of calibrated instrumentation the force in grams applied, up until the animal withdraws its limb, is recorded. Male C57BL6 mice (n=16, 23-27g) were induced with a mild unilateral inflammation of the left knee joint (Gauldie *et al.*, 2004), which resulted in chronic joint hypersensitivity. PAD withdrawal thresholds were measured prior to and for 21 days following induction of unilateral joint inflammation. On days 13-17 the animals were dosed with the steroid; prednisolone (1mg.kg⁻¹), which is clinically effective in the treatment of osteoarthritis. We found, that PAD accurately detected the initial joint hypersensitivity and the subsequent analgesic action of prednisolone in mice. We conclude that PAD is a robust device which provides an easy to use objective method for assessing localised joint hypersensitivity, humanely, in laboratory animals. PAD is a new behavioural tool which will facilitate the development and screening of novel analgesics for clinical use.

Gauldie *et al.*, 2004 *J Neurosci Methods* **139**(2):281-291

Pressure Application Device (PAD) – A novel behavioural readout for assessing a localised hypersensitivity of the knee joint in a murine model of unilateral hypersensitivity

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Aim

The aim of this study was to develop and validate a novel quantitative behavioural readout in a mouse model of unilateral hypersensitivity (Gauldie *et al.*, 2004) which mimics mild arthritis in man. The Pressure application device (PAD), is the first mechanical tool designed to objectively assess a localised hypersensitivity directly across the knee joint. We aimed to validate this device in order to improve the translation of novel analgesics from *in vivo* models into man.

Introduction

Osteoarthritis (OA) and Rheumatoid arthritis (RA) are highly prevalent inflammatory conditions; with OA having an estimated incidence of 12.5% and RA 0.8% in the UK adult population (Bedson *et al.*, 2005). With current treatments providing poor pain relief and commonly associated with undesirable side effects, there still remains a great demand for the development of new, safer analgesics. Several behavioural readouts are currently used to assess the hypersensitivity in the joints of arthritic rodents (Clayton *et al.*, 1997). However none of these are able to assess a localised hypersensitivity directly across the arthritic joint.

Apparatus

PAD apparatus includes a base unit with display screen (Fig.1A) and a force transducer, with 5mm diameter application surface, mounted on a unit worn on the experimenters thumb. Placing the force transducer on one side of the joint and the forefinger on the opposite (Fig. 1B), a gradually increasing 'squeeze' was applied in a mediolateral plane across the mouse knee joint. By means of the calibrated instrumentation the force in grams (gf) applied was recorded (max = 500gf). The test endpoint was when the animal withdrew its limb. On test days three recordings were taken per animal in order to provide a mean daily withdrawal threshold value.

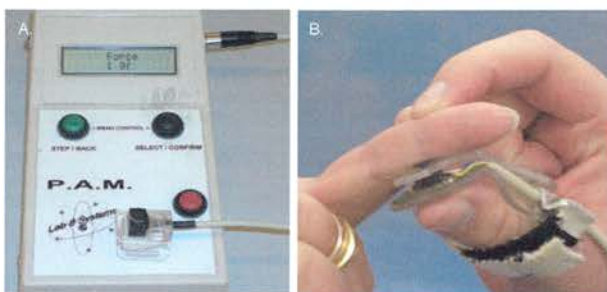


Fig. 1. (A) PAD apparatus showing the base unit with force display screen and thumb worn force transducer. (B) Example of how the gradually increasing 'squeeze' force is applied across a joint. The transducer is placed on one side of the joint and the forefinger on the opposite.

Methods

Experiments were performed in accordance with Home Office regulations and within UK animal welfare guidelines, and received Local Ethics Committee approval. Male C57 black 6 mice (24, C57BL/6; Charles River, UK; 23-28g) were used in the study. Mice were housed 8 to a cage in a 12-h light/dark environment and were given free access to standard animal feed and water for the duration of the study. All animals were allowed three days acclimatisation before the experiment began and all behavioural tests took place in the same room at the same time of day.

A chronic inflammatory joint pain was induced following day 0 baseline PAD withdrawal threshold recordings. Mice were transiently anaesthetised (3% isoflurane with O₂ at 1.5l.min⁻¹) and a small incision was made over the knee joint to expose the patella tendon, sham (n=8) animals recovered with no further treatment. The remaining 16 animals were injected with Freund's complete adjuvant (FCA; 20µl, 10mg.ml⁻¹) under the patella tendon into the intra-articular space of the knee joint (Gauldie *et al.*, 2004).

On day 10 the FCA injected animals were ranked and randomised into two matched groups (n=8 per group), based on their level of withdrawal threshold (gf). The groups were dosed (s.c.) on days 13-17 blindly with either prednisolone (1mg.kg⁻¹) or vehicle (ethanol 5%, PEG 45% and distilled water 50%) one hour before recordings were taken.

Data were transformed into an area under the curve (AUC) value during the dosing period and analysed using a Mann-Whitney test, where $P < 0.05$ was considered significant.

Results

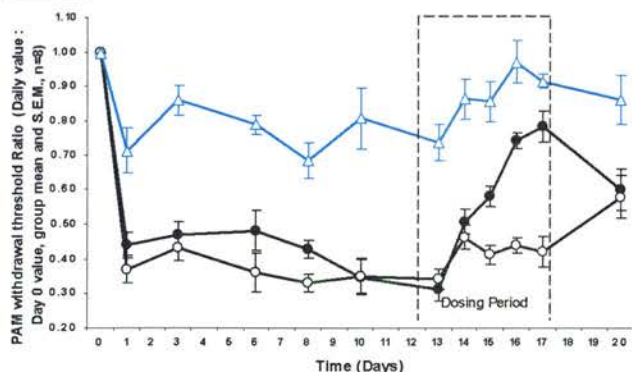


Fig. 2. Effect of prednisolone (n=8, s.c., 1mg.kg⁻¹, filled circle) or vehicle (open circle) on the FCA induced hypersensitivity (n=16, 1 ant., 20µl, all circles) over days 13-17 as measured by PAD. Untreated sham (n=8) animals are represented by open triangles. Day 0 ratios of 1.0 are based on the day 0 group mean withdrawal threshold values of 402gf, 400gf and 437gf.

• FCA injected animals had significantly lower withdrawal thresholds than sham on day 3 ($P < 0.01$), compared to day 0, confirming FCA induced a localised hypersensitivity which was detected using PAD.

• During the treatment period (days 13-17) the prednisolone treated group had significantly higher AUC values, compared to day 10, than the vehicle group ($P = 0.0104$). This was due to a prednisolone induced reversal of hypersensitivity.

Summary and Conclusions

• PAD is a robust instrument which provides an easy to use, objective method for localised assessment of knee joint hypersensitivity in mice. PAD will facilitate the screening and development of novel analgesics for use in the clinic.

• A second PAD model is currently under development designed and calibrated for measuring hypersensitivity in rat knee joints.

Acknowledgements

Dr. Harry Brash for his hard work in designing and building PAD. The work was funded by GlaxoSmithKline and the BBSRC as part of PhD studies (ITS and NJB).

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Na_v1.9 EXPRESSION IN A DISTINCT POPULATION OF KNEE JOINT AFFERENTS, IN A RAT MODEL OF CHRONIC JOINT INFLAMMATION

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The expression of the sodium channel Na_v1.9 has been shown to be altered in animal models of neuropathic and acute inflammatory pain (Tate et al., 1998) and its role has been implicated in the maintenance of inflammatory hypersensitivity (Priest et al., 2005). The aim of this study was to profile changes in Na_v1.9 expression over a 28 day time course in a rat model of chronic inflammatory joint pain (Wilson et al., 2006). We used the fluorescent retrograde tracer Fast Blue (FB) to highlight a discrete population of knee joint afferents identifying a specific DRG neuronal profile.

Male Wistar rats (Charles River, n=54, 150-200g) were transiently anaesthetised using 3% isoflurane in oxygen, and FB (10 μ l, 20mg.ml⁻¹) was injected into the joint space of the left knee. One week later test animals received an injection of Freund's Complete Adjuvant (FCA; 150 μ l, 1mg.ml⁻¹) into the same joint to induce chronic inflammatory joint hypersensitivity. Control animals received no FCA. Animals (FCA and control, n=6 per group) were culled on days 7, 14, 21 and 28 post-FCA, and ipsilateral L3-L5 DRGs were removed, immediately snap frozen and processed for fluorescent immunohistochemistry. Serial 12 μ m cryosections were cut, fixed in paraformaldehyde and labelled overnight with Na_v1.9 primary antibody (GSK in house, 1:200). Sites of antibody binding were revealed using the secondary antibody; goat anti-rabbit-Alexa 488 (Molecular Probes, 1:200). In 5-7 sections of each DRG, the total number of FB positive cell bodies was counted, as was the number of those FB positive cell bodies which also expressed Na_v1.9. The group mean co-localisation percentage (\pm SEM) was calculated from the total counts of L3-L5 for each animal (see Figure 1).

At days 14, 21 and 28 there was a significant increase in the percentage of Na_v1.9 positive knee joint afferent cell bodies innervating the hypersensitive joints (Mann Whitney, $P<0.05$ vs. control). These results provide further evidence that Na_v1.9 channels may play a role in maintaining neuronal hyperexcitability in chronic inflammatory joint pain.

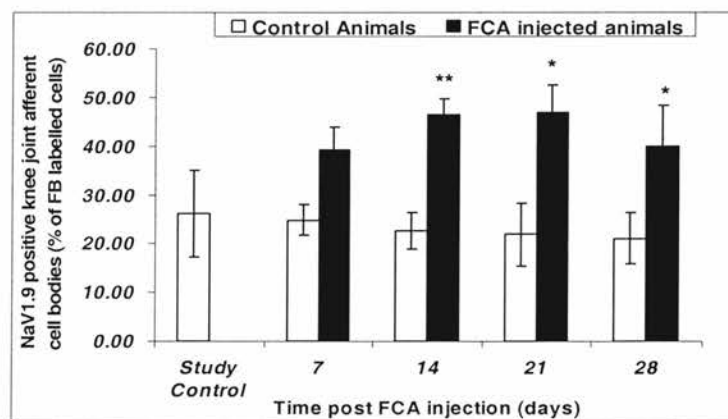


Figure 1. Percentage of Na_v1.9 positive knee joint afferent cell bodies in control and FCA-induced knee joint hypersensitive animals over a 28 day time course study (n=6 per group, * = $P<0.05$, ** = $P<0.01$, Mann Whitney test)

Priest et al., 2005 *Proc.Natl.Acad.Sci.U.S.A* **102**, 9382-9387.

Tate et al., 1998 *Nat Neurosci* **1**, 653-655.

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Na_v1.9 Expression in a distinct population of knee joint afferent cell bodies, during a rat model of chronic joint inflammation

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Introduction

The expression of the sodium channel Na_v1.9 has been shown to be altered in animal models of neuropathic and acute inflammatory pain (Tate et al., 1998) and its role has been implicated in the maintenance of inflammatory hypersensitivity (Priest et al., 2005). These TTX-resistant sodium channels are expressed in the dorsal root ganglion (DRG) where they can be transported centrally, or to the periphery to regulate neuronal hyperexcitability.

In the present study we used a Freund's complete adjuvant (FCA) insult to establish chronic inflammatory hypersensitivity of the knee joint (Wilson et al., 2006). By using the retrograde tracer fast blue (FB) we identified a distinct population of cell bodies of knee joint afferent nerves (Hanesch et al., 1995) and subsequently investigated Na_v1.9 receptor expression levels in this specific neuronal population in ipsilateral L3-L5 DRGs, for 28 days post-FCA.

Methods

Study Design

All experiments were performed using adult male Wistar rats (Charles River, UK; n=54) weighing between 150–200g. Prior to FB injection, and throughout the study, hind limb weight distribution was measured using the Linton Incapacitance tester (Wilson et al., 2006).

For intra-articular knee joint injections of FB and FCA, animals were transiently anaesthetised using 3% isoflurane in oxygen, the left (ipsilateral) knee joint was shaved and cleaned and 10 µl of FB (20 mg ml⁻¹, Illing Plastics, Germany) or 150 µl of FCA (1 mg ml⁻¹, Sigma, UK) was injected under the patella tendon into the joint space of the left knee. Animals recovered from anaesthesia before being returned to their cage.

On day minus 7, all animals (n=54) received an initial injection of FB. 7 days later (day 0), test animals (n=24) received a further injection of FCA, whilst control animals (n=30) received no further treatment. Animals were killed (FCA and control, n=6 per group) on days 7, 14, 21 and 28 post FCA. Ipsilateral L3-L5 DRGs were removed and embedded in optimum cutting temperature (TissueTek, Canada) compound and snap frozen in isopentane cooled on dry ice. DRGs were stored at -80°C until they were sectioned.

Fluorescent Immunohistochemistry

Serial 12 µm cryosections were cut, fixed in paraformaldehyde and labelled overnight with Na_v1.9 primary antibody (GSK in house, 1:200). Sites of antibody binding were revealed using the secondary antibody; goat anti-rabbit-Alexa 488 (Molecular Probes, 1:200).

Analysis

In 5–7 sections of each DRG, the total number of FB positive cell bodies was counted, followed by the number of those FB positive cell bodies also expressing Na_v1.9. The group mean co-localisation percentage (± SEM) was calculated from the total counts of L3-L5 for each animal.

Results

1. FCA induced a significant hypersensitivity up to 28 days post injection

Injection of fast blue on day minus 7 had no effect as assessed using the weight distribution ratio (see Figure 1.)

Figure 1.

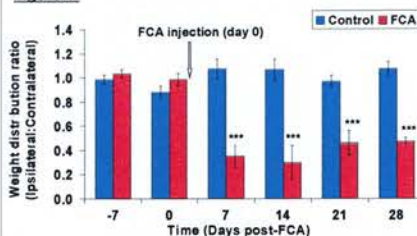


Fig. 1. Time course study behavioural data – intra-articular injection of FCA caused a significant hypersensitivity at all timepoints (n=54, Mean ± SEM, ANOVA followed by Bonferroni's post-hoc comparisons, *** = P<0.001).

2. Fast Blue acts as a retrograde tracer, labelling the cell bodies of the knee joint afferents

Figure 2.

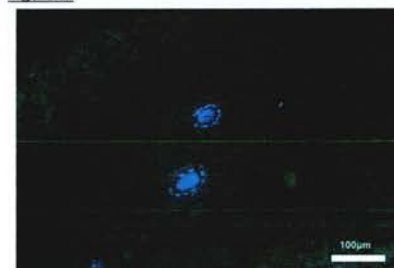


Fig. 2. Sample photomicrograph showing the selective fluorescence of fast blue positive knee joint afferent soma.

Following intra-articular injection of 10 µl of 2% fast blue, afferents retrogradely transport the dye back to their somata located in the DRG. Using fluorescent microscopy these fast blue-positive cells are clearly visible, highlighting the cell bodies of nerves innervating the knee joint.

3. The majority of knee joint afferents have their cell bodies in L3 and L4 of DRG tissue

Figure 3.

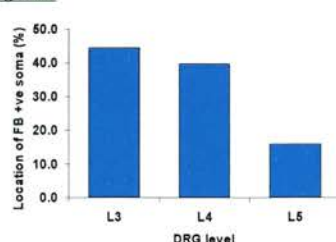


Fig. 3. Distribution of fast blue-positive cell bodies in L3-L5 DRGs.

A total of 788 fast blue-positive cell bodies was counted from L3-L5 ipsilateral DRGs. Of these cells 84% were found in L3 and L4. These results are in accordance with previously published data (Salo et al 1997), where 88% of knee joint afferent cell bodies were found in L3 and L4 when looking at L1-L5 rat DRGs

4. Na_v1.9 receptor expression increases 14, 21 and 28 days post-FCA

Figure 4.

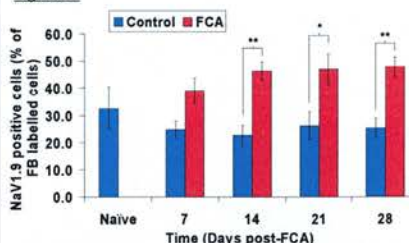


Fig. 4. FCA caused an increase in the percentage of Na_v1.9 receptor expressing knee joint afferent cell bodies at 14, 21 and 28 days post FCA (Mean ± SEM, Mann Whitney FCA vs. control, * = P<0.05 and ** = P<0.01).

Figure 5.

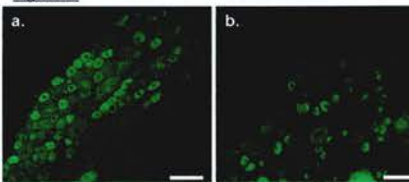


Fig. 5. Example photomicrographs of Na_v1.9 staining in a. FCA and b. control DRG sections at day 21. Scale bar represents 100 µm

Conclusions

- FCA causes behavioural hypersensitivity that is established by day 7 and maintained at day 28 post-injection. This behavioural hypersensitivity is mirrored by an increase in Na_v1.9 receptor expression in cell bodies of knee joint afferents over this time period.
- The increased receptor expression is likely to lead to a shift in the resting membrane potential closer to the activation threshold and increase neuronal excitability.
- These results provide further evidence that Na_v1.9 channels play a role in the maintenance of neuronal hyperexcitability in chronic inflammatory joint pain.

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Acknowledgements



Peripherally administered endomorphin-1 attenuates nociceptive discharge evoked by noxious mechanical stimulation in a rat model of chronic inflammatory joint hypersensitivity

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Endomorphin-1 (EM-1) is an endogenous tetrapeptide with high selectivity and affinity for the μ -opioid receptor (Zadina et al., 1997). EM-1 has similar functional properties to morphine and has been shown to inhibit nociceptive activity in primary afferents during noxious mechanical hyper-rotation of acutely inflamed knee joints (Li et al., 2005). The aim of this in vivo electrophysiology study was to determine whether EM-1 could attenuate mechanical joint hypersensitivity 14-23 days after induction of an inflammation in the knee joint (Donaldson et al., 1993).

Unilateral knee joint arthritis was induced in the left joint of adult male Wistar rats (n=15, 150-200g) by a 150 μ l intra-articular injection of Freund's complete adjuvant (FCA; 1mg.ml⁻¹ Mycobacterium tuberculosis, Sigma, UK). Rats were transiently anaesthetised using 3% isoflurane in oxygen and FCA was injected into the knee joint space. On days 14-23 animals were anaesthetised by an i.p. injection of pentobarbital sodium (60mg.ml⁻¹ at 10 μ l.kg⁻¹), maintained throughout the experiments by an i.v. infusion of pentobarbital (7.8mg.hr⁻¹). Extracellular neuronal recordings were made from the medial articular nerve (MAN) of the ipsilateral knee joint (Gauldie et al., 2001). The MAN was mechanically stimulated using von Frey filaments of 1g, 7g and 21g at a fixed position within the receptive field on the exposed surface of the knee joint. Filaments were tested in ascending order for a period of 10s with a break of 10s in between each stimulation. Applications of the hairs were repeated every 20mins. The effect of each individual filament was measured as the change in the action potential frequency (impulses.s⁻¹), during the test period compared to that of the preceding ten seconds. Five tests were performed before and after dosing with EM-1 (100 μ g, n=5), naloxone (500 μ g) and EM-1 (100 μ g, n=5) and saline vehicle (n=5). Drugs were administered in a volume of 100 μ l close to the knee via a femoral arterial cannula. The mean (\pm SEM) normalised frequency of firing was calculated for each individual filament, with the highest frequency within each experiment being designated 100%. Data were analysed using a one-way analysis of variance (ANOVA, Kruskal Wallis) where $P < 0.05$ was considered statistically significant.

Results showed that EM-1 significantly inhibited firing of the MAN evoked by the 7g and 21g von Frey filaments. The mean discharge evoked by the 7g filament fell significantly from 6.7 ± 1.6 impulses.s⁻¹ to 1.3 ± 0.3 impulses.s⁻¹ ($P < 0.05$, $46 \pm 5\%$ to $10 \pm 2\%$) and the firing frequency evoked by the 21g filament fell significantly from 9.7 ± 2 impulses.s⁻¹ to 2.2 ± 0.4 impulses.s⁻¹ ($P < 0.05$, $70.8 \pm 5\%$ to $24 \pm 5\%$). This significant inhibition was reduced when naloxone was administered before the EM-1. The vehicle had no significant effect on the frequency of firing evoked by any of the filaments tested. These results suggest that μ -opioid receptors are present and functional in the periphery on the MAN during the advanced stages (days 14-23) of this model of chronic inflammatory joint hypersensitivity. This is further evidence that peripherally acting opioids could play an important role in managing chronic pain in the clinic, without the unwanted side effects of the centrally acting opioid morphine.

Donaldson, L. et al., (1993) *J Neuro Methods* **49**, 5-10

Gauldie, S. et al., (2001) *Br J Pharmacol* **132**, 617-21

Li, Z. et al., (2005) *Arthritis Rheum* **56**, 3210-3219

Zadina, J. et al., (1997) *Nature* **386**, 499-502

Peripherally administered endomorphin-1 attenuates nociceptive discharge evoked by noxious mechanical stimulation in a rat model of chronic inflammatory joint hypersensitivity



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1. Aims of the study

An *in vivo* electrophysiology study was undertaken to investigate the effect of endomorphin-1 (EM1) on the neural activity of nociceptors in the rat medial articular nerve (MAN), as assessed by the response to noxious mechanical stimulation 14-23 days after the induction of an inflammation in the knee joint.

2. Introduction

EM1 is an endogenous tetrapeptide with a high selectivity and affinity for the μ -opioid receptor (MOR). EM1 has been shown to exhibit analgesic properties similar to morphine (Zadina et al., 1997).

Experimental inflammation of the rat knee joint, induced by Freund's complete adjuvant (FCA), is a model of human inflammatory arthritis and has been used to investigate the mechanisms involved in the establishment of chronic pain (Donaldson et al., 1993). Moreover the model has been utilised as a pre-clinical screen for novel analgesics in a drug discovery environment.

EM1 has been shown to inhibit nociceptive activity in primary afferent fibres of the MAN in response to noxious hyper-rotation of the FCA-inflamed knee joint. This effect is present in naive animals and animals tested at an acute stage of the model (48hrs post-FCA), however the effect is absent in animals tested 7 days into the model, leading the authors to conclude that MOR are down-regulated during chronic inflammation (Li et al., 2005).

Intra-articular injection of morphine is known to produce a long lasting analgesia in humans suffering from inflammatory arthritis in the knee (Likar et al., 1997). With this in mind the present study was performed in order to investigate whether functional MOR are present in the knee joint primary afferents during a more chronic phase of the FCA-induced rat model of joint inflammation than has previously been studied.

3. Methods

Unilateral arthritis was induced in the left knee joint of adult male Wistar rats ($n=15$, 150-200g, Charles River) by an intra-articular injection of 150 μ l of FCA (1mg.ml⁻¹ Mycobacterium tuberculosis, Sigma). Rats were transiently anaesthetised using 3% isoflurane in oxygen and FCA was injected into the knee joint space. On days 14-23 animals were anaesthetised by an i.p. injection of pentobarbital sodium (60mg.ml⁻¹ at 10 μ l.kg⁻¹), maintained throughout the experiments by an i.v. infusion of pentobarbital (7.8mg.hr⁻¹). Extracellular neuronal recordings were made from the MAN of the left knee (Gauldie et al., 2001).

The MAN was mechanically stimulated using von Frey filaments of 1, 7 and 21g at a fixed position within the receptive field of the MAN, on the exposed surface of the knee joint. Filaments were tested in ascending order for a period of 10s with a break of 10s between successive stimuli. Tests were conducted every 20mins, with five tests being performed before and after dosing with either vehicle (saline, $n=5$), EM1 alone (100 μ g, $n=5$), or EM1 (100 μ g, $n=5$) following pre-treatment with 500 μ g naloxone (see Figure 1). Drugs were administered in a volume of 100 μ l close to the knee via an arterial femoral cannula.

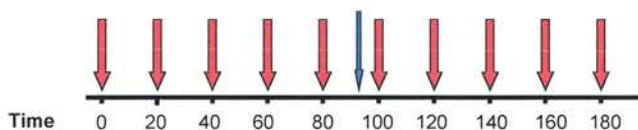


Figure 1. Schematic of the test protocol showing the times of mechanical stimulation (red arrows) and the time when the drugs were delivered (blue arrow). Pre-treatment with naloxone (500 μ g) occurred 3 minutes before the EM1 was administered.

The effect of each individual filament was measured as the change in the action potential frequency (impulses.s⁻¹) during the test period compared to that of the preceding ten seconds. The mean (\pm SEM) neural discharge was calculated for each individual filament. Data were analysed using a one-way analysis of variance (ANOVA, Kruskal Wallis) where $P<0.05$ was considered statistically significant.

4. Results

EM1 inhibited firing of the MAN evoked by the 1, 7 and 21g von Frey filaments. The mean neural discharge fell from 0.6 ± 1.6 to 0.09 ± 0.03 impulses.s⁻¹ evoked by the 1g filament, 3.3 ± 0.6 to 0.64 ± 0.2 impulses.s⁻¹ evoked by the 7g filament and 4.8 ± 0.7 to 1.1 ± 0.2 impulses.s⁻¹ evoked by the 21g filament, when comparing pre-drug values to post-drug values. All the reductions in neural firing were significant (* represents $P<0.05$, *** represents $P<0.001$, Kruskal Wallis ANOVA). This significant inhibition was not observed when naloxone was administered 180s before EM1. The vehicle (saline) had no effect on the frequency of firing evoked by any of the filaments tested (see Figure 2).

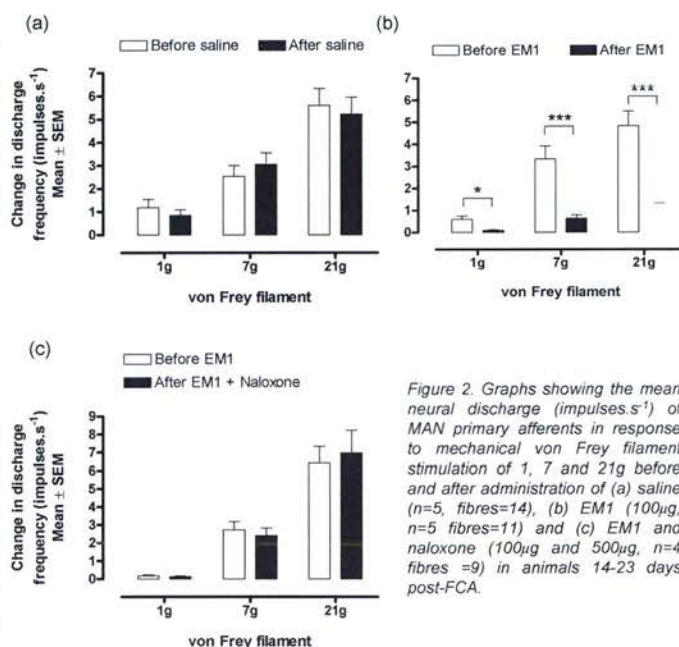


Figure 2. Graphs showing the mean neural discharge (impulses.s⁻¹) of MAN primary afferents in response to mechanical von Frey filament stimulation of 1, 7 and 21g before and after administration of (a) saline ($n=5$, fibres=14), (b) EM1 (100 μ g, $n=5$ fibres=11) and (c) EM1 and naloxone (100 μ g and 500 μ g, $n=4$ fibres=9) in animals 14-23 days post-FCA.

5. Conclusions

- These results suggest that MOR are present and functional in primary afferent nerves during the advanced stages (days 14-23) of the FCA-induced model of chronic inflammatory joint hypersensitivity.
- The potent antinociceptive effect of the MOR agonist EM1, occurs through an opioid receptor mediated mechanism, as the antinociceptive effect is blocked by pre-treatment with the opioid receptor antagonist naloxone.
- This study provides further evidence that peripherally acting opioids play an important role in managing chronic pain in the clinic, without the unwanted side effects of the centrally acting opioid morphine.

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Pressure application measurement (PAM): A novel behavioural technique for measuring hypersensitivity in a rat model of joint pain

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Abstract

Chronic joint pain affects physical well being and can lead to severe psychological and social problems, therefore successful long-term management is highly sought-after. No current behavioural measures of pain used in pre-clinical models mimic the clinical dolorimeter, which provides an objective measure of joint hypersensitivity. In this study we aim to use a novel behavioural readout alongside an established measure to mimic the multifactorial measurements taken in the clinic. Using the pressure application measurement (PAM) device a gradually increasing squeeze was applied across the knee joint of rats until the animal gave an indication of pain or discomfort. PAM and the incapitance tester were used to detect joint hypersensitivity in a well-established rodent model of adjuvant-induced arthritis. Subsequently, the analgesic effects of prednisolone (1, 3 or 10 mg kg⁻¹), morphine (3 mg kg⁻¹) and celecoxib (15 mg kg⁻¹) were assessed. Both PAM and the incapitance tester detected a reversal of hypersensitivity 1 h post-drug administration. Furthermore, the two readouts were highly correlated, and power analysis indicated that PAM was highly reproducible. In conclusion, PAM provides a novel, accurate behavioural tool for detecting a primary mechanical hypersensitivity in a rat model of chronic inflammatory joint pain.

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Keywords: Rat; Hypersensitivity; Joint; Force; Withdrawal; Pressure application

1. Introduction

Osteoarthritis (OA) and rheumatoid arthritis (RA) are prevalent diseases, with estimated incidences of 12.5% (Bedson et al., 2005) and 0.8% (Symmons, 2005) in the UK adult population, respectively. Arthritis is associated with chronic, debilitating pain in the joints. Pain is the most common symptom of patients seeking medical consultation (Loeser and Melzack, 1999). Chronic joint pain not only affects physical well being, for instance impairing the ability to work, but can also lead to severe psychological and social problems as a result of sleep disturbance, anxiety and depression (Ashburn and Staats, 1999). Successful long-term management is therefore highly sought-after.

Rodent models of chronic inflammatory joint hypersensitivity have been developed (Chillingworth and Donaldson, 2003; Donaldson et al., 1993; Gaudie et al., 2004; Wilson et al., 2006) to help elucidate the underlying pathophysiology involved in arthritic conditions by identifying specific key modulators or receptors involved in the pain process. These models are commonly used as pre-clinical screens for novel analgesics, with the aim to improve the treatment of chronic joint pain in the clinic. In order to do this successfully it is essential that results from pre-clinical models correlate well with observed changes in the clinic to facilitate the translation of novel treatments into successful patient pain management.

To date, several behavioural readouts have been used to assess joint hypersensitivity in animal models, using thermal, mechanical, or electrical sensory stimuli (for review see Le Bars et al., 2001). Only the weight distribution readout obtained using an incapitance tester (Clayton et al., 1997) has so far been considered as a fair measurement of the persistent chronic hyper-

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sensitivity observed in patients. The incapacitance tester is able to provide an objective, non-evoked assessment of “incident” pain by measuring the average weight placed on each hind limb. Naïve rodents distribute their weight equally between both hind limbs under normal conditions (Kobayashi et al., 2003). However, following induction of joint inflammation, animals redistribute their weight in order to put less weight through the affected joint.

Whilst this readout has gone a long way to correlate with the clinical observation of ‘pain on standing’ it is only one measurement of complex pain. In the clinic, patients are scored by a variety of readouts or tasks, often being subjected to firm squeezing of affected joints (Ritchie et al., 1968). For instance, using the pressure dolorimeter (Langley et al., 1983), a localised quantitative measure of hypersensitivity directly around the affected joint can be measured. Using this device, a gradually increasing pressure is applied perpendicularly across the joint until the patient indicates tenderness or pain, at which point a ‘sensitivity’ score is recorded. Clinicians are able to summate a number of quantitative evaluations to assess a patient’s level of pain or discomfort. In this study we aim to use a novel behavioural readout alongside an already established readout, with a view to bringing together two quantitative animal evaluations to mimic the multifactorial measurements taken in the clinic.

Here we describe a novel behavioural readout, for use with rats, utilising similar principles to the clinical pressure dolorimeter. The pressure application measurement (PAM) device is designed to apply a gradually increasing squeeze pressure directly across the knee joint of rats until the animal gives an indication of pain or discomfort. At this point a quantitative value of the force applied is recorded, giving a similar ‘sensitivity’ score.

The aim of this study was to determine if PAM was able to detect a Freund’s complete adjuvant (FCA)-induced hypersensitivity in the knee joints of rats, and subsequently if PAM could detect a reversal of this hypersensitivity using analgesics known to be effective in the clinic. A dose–response study using prednisolone (Pyne et al., 2004), and further studies using morphine (Caldwell et al., 2002), and celecoxib (Schnitzer et al., 2005), were performed to investigate the reproducibility and sensitivity

of PAM, whilst comparing the device with the already established weight distribution readout. Thus enabling an additional, clinically relevant pain readout to be applied pre-clinically aiding the translation of novel analgesics from animal to man.

2. Materials and methods

Experiments were performed in accordance with Home Office regulations and within UK animal welfare guidelines, and received Local Ethics Committee approval. Adult male Wistar rats (Charles River, UK; initial weight range 137–284 g) were used in the study. Animals were housed four to a cage in a 12 h light/dark environment and were given free access to standard animal feed and water for the duration of the study. All animals were allowed 6 days acclimatisation before the experiment began and all behavioural tests took place in the same room at the same time of day. Animals were weighed on each test day to monitor their general health.

2.1. Behavioural assessment

2.1.1. PAM–knee joint withdrawal threshold measurement

PAM consists of a force transducer mounted on a unit fitted to the operator’s thumb (see Fig. 1). The thumb unit is connected to a recording base unit containing the control panel and digital readout display. The apparatus has a force transducer with a range of 0–1500 g (Honeywell, Farnell, UK) and the diameter of the circular contact is 8 mm; giving the device a surface area of 50.3 mm².

Animals were lightly, but securely held and the operator placed the thumb unit on one side of the animal’s knee joint and the forefinger on the other. A gradually increasing squeeze force was applied across the joint at a rate of approximately 300 grams per second ensuring the maximum test duration was 5 s. By means of calibrated instrumentation the force in grams applied was displayed on the digital screen and recorded. The test endpoint was when the animal withdrew its limb or showed any behavioural signs of discomfort or distress, such as freezing of whisker movement or wriggling. As the animals were lightly

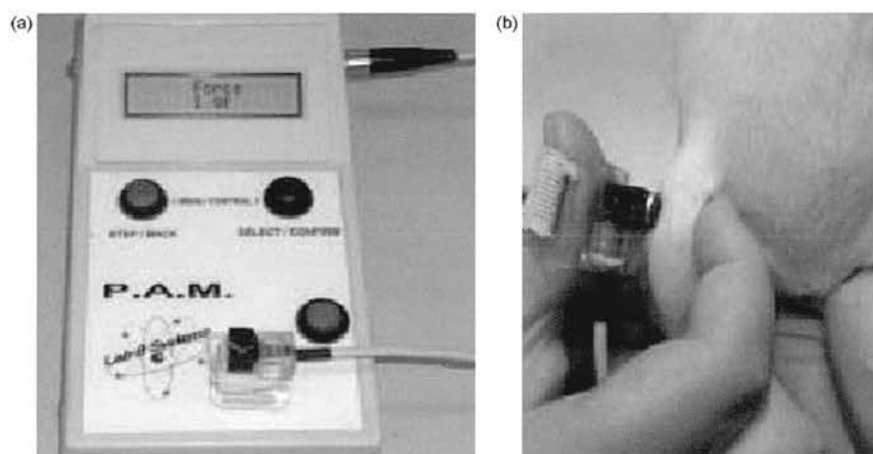


Fig. 1. Presentation of the pressure application measurement (PAM) device. (a) The portable control unit with digital display and thumb attachment; (b) PAM in use measuring the limb withdrawal threshold (LWT) of a rat.

held with their hind limbs suspended, any movements to pull or twist out of the operator's fingers were obvious. Any motion to attempt to withdraw from the device was transferred from the rat limb to the operator's fingers and forearm, and was taken as an indication of the test end point. On very rare occasions the first sign of distress was shown as the animal vocalising prior to limb withdrawal, and on these occasions this was taken as the test end point. The peak gram force (gf) applied immediately prior to limb withdrawal was recorded by the base unit, and this value was designated the limb withdrawal threshold (LWT). Three measurements of both the ipsilateral and contralateral limbs were made at 1 min intervals during which the animals were returned to their respective cages. The mean LWTs were calculated.

2.1.2. Incapacitance tester—weight distribution

The weight distribution between the animal's hind limbs was measured using the incapacitance tester (Linton Instruments, UK). The apparatus consists of two force transducers capable of measuring the body weight that the animal places on each hind limb. Animals were placed on the incapacitance tester with their hind paws centred on the two force transducers; the average body weight distribution in grams was calculated over a period of three seconds. Weight placed through the ipsilateral limb was expressed as a ratio of the weight placed through the contralateral limb, with a ratio of 1.0 resulting from equal weight distribution across both hind limbs. Each animal had three recordings taken per test day and the mean weight distribution ratio was calculated for each group.

2.1.3. Knee joint diameter

The knee joint diameters (left and right) were measured just below the level of the patella using hand-held digital micro-callipers (Mitutoyo, Japan; accuracy ± 0.1 mm), used as a measure of swelling during the study.

2.2. Arthritis induction

Rats were transiently anaesthetised with 3% halothane in oxygen. FCA (1 mg ml⁻¹; Sigma, UK) was injected at a volume of 150 μ l into the joint space of the left knee through the patella tendon using a sterile 25-gauge needle (B&D Microlance, UK). Control animals were anaesthetised but received no injection. The animals were allowed to recover from anaesthesia before being returned to cages.

2.3. Study design and drug treatment

A pilot study was carried out to determine whether PAM could detect a window of hypersensitivity following intra-articular FCA in comparison with sham animals. The study also compared values for PAM with those obtained in the same animals using the incapacitance tester. Baseline values for body weight, weight distribution, PAM LWT and knee joint diameters were measured on day 0, prior to induction of the joint inflammation, and were repeated two to three times per week until day 28. No habituation to the PAM device was conducted.

Secondly, a drug-treatment study was carried out, to investigate whether PAM was able to detect reversal of this joint hypersensitivity and how this differed with three types of drug, an opioid, a steroid and a non-steroidal anti-inflammatory drug (NSAID). In order to perform a thorough pharmacological investigation in this study, whilst using the minimum number of animals, a dose-response relationship to prednisolone was examined and single top doses of an opioid and an NSAID were also investigated. A pre-study behavioural assessment was introduced to habituate the animals to the device. Baseline values for body weight, weight distribution, PAM LWT and knee joint diameters were measured on day 0, prior to induction of the joint inflammation. Measurements were made two to three times per week until day 10, when animals were randomly assigned into treatment groups. Drugs were administered subcutaneously (s.c.) in a volume of 2 ml kg⁻¹ between days 14 and 18. Prednisolone (1, 3 or 10 mg kg⁻¹) was dosed once per day; whereas morphine (3 mg kg⁻¹), celecoxib (15 mg kg⁻¹) and vehicle (ethanol 5%, PEG 45% and distilled water 50%) were given twice a day. The dose range of prednisolone used was decided from published results from the lab (Gauldie et al., 2004), and single doses of morphine and celecoxib were decided from other studies giving strong positive reversals of hypersensitivity (Wilson et al., 2006). One hour after blind dosing, behavioural assessments were made following a strict protocol in order to give animals a rest period between the weight distribution and the PAM measurements. Briefly, animals were weighed and had their weight distribution assessed by making three consecutive readings before being returned to their cage. Ten minutes later animals had the sensitivity of both the ipsilateral and contralateral joints assessed using PAM. Three consecutive readings were taken, with a 1 min interval between each measurement, where the animal was returned to the cage. Finally the knee joint diameters were measured. Further behavioural assessments were carried out once the dosing period was complete, on days 21, 24 and 28.

2.4. Data analysis

Prior to analysis, an area under the curve (AUC) summary of each animal's repeatedly measured responses during the dosing period was calculated. These AUC values were then analysed using a one-way analysis of variance (ANOVA) approach. Measurements taken over several days were analysed using a repeated measures ANOVA. In both cases, individual groups were compared using planned comparisons on the predicted means. Differences between ipsilateral and contralateral sides were analysed using paired *t*-tests. In all cases the null hypothesis was rejected at $P < 0.05$. The data were log transformed where appropriate to stabilize the variance. Data are expressed as observed mean \pm standard error of the mean (SEM). Statistical analyses were carried out using Statistica version 6 (Statsoft, Tulsa, OK, USA).

Two different power analyses were carried out on the drug study to investigate the reliability and confidence levels obtained from the two different readouts in the framework of this experimental design. In the first, perhaps more standard power

analysis, the number of animals required to reach a certain power obtained from an observed drug effect was assessed. For this analysis, calculations were based on an effect size equivalent to the observed difference between vehicle and the highest dose of prednisolone.

In the second analysis, the effect of varying the number of animals used, or the numbers of measurements made, on the power of the experiment was assessed. By calculating the animal and measurement variance components, for the weight distribution and PAM LWT responses separately, we investigated the effect of varying replication of both animals and the measurements on the statistical power of the treatment comparisons.

3. Results

In both studies, intra-articular injection of FCA had no adverse effects on the animal's general health as evidenced by the fact that they continued to feed and gain weight normally throughout the study. There were no significant differences in body weights between any of the FCA-injected and sham animals at any time point (data not shown).

In both studies there were no significant differences between the mean PAM LWTs or knee joint diameters of the ipsilateral and contralateral limbs and no significant differences between treatment groups, prior to FCA injection on day 0 ($P > 0.05$).

3.1. Behavioural evaluation of FCA-induced hypersensitivity

3.1.1. Pressure application measurement (PAM)

Prior to induction of joint inflammation (on day 0), the average ipsilateral LWT was 710 ± 41 gf ($n = 16$) and the average contralateral LWT reading was 790 ± 39 gf ($n = 16$). Following injection of FCA, the average ipsilateral LWT of the treatment group decreased by 57% to 316 ± 45 gf ($n = 8$), by day 1. This was significantly lower than both the day 0 value ($P < 0.05$) and the sham group at the same time point ($P < 0.001$; see Fig. 2a). Despite receiving no further treatment the ipsilateral LWT of sham animals increased significantly by 57% to 1028 ± 48 gf ($n = 8$, $P < 0.05$) on day 1. The average ipsilateral LWT of FCA-injected rats were significantly lower than sham and contralateral joints ($P < 0.05$) over the full 28-day time course studied.

3.1.2. Incapacitance tester—weight distribution

The average ratio of weight distribution between ipsilateral and contralateral limbs in rats was 0.96 ± 0.03 ($n = 16$) on day 0. The ratio of weight distribution between the hind limbs for FCA injected rats decreased by 70% to 0.29 ± 0.05 ($n = 8$, see Fig. 2b). This was significantly less than sham animals ($P < 0.05$), which had a ratio of 1.05 ± 0.02 ($n = 8$) at this time point. The ratio remained significantly reduced in FCA-injected animals compared with the sham group up to and including day 28 ($P < 0.001$). There were no significant changes observed in the sham group at any time point in this behavioural readout.

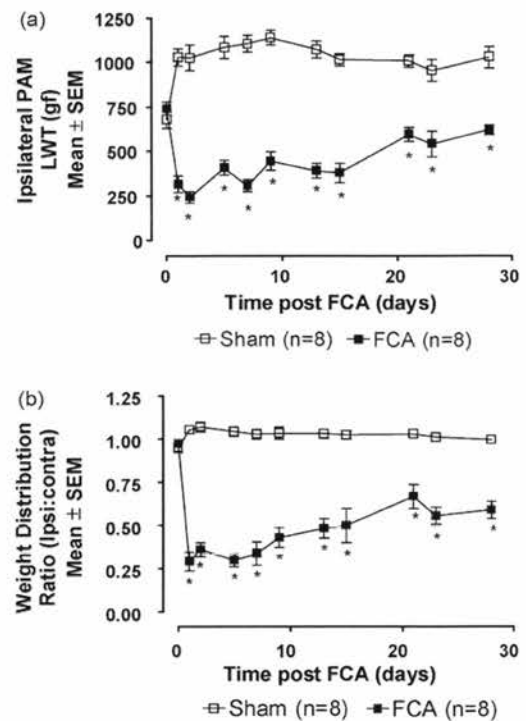


Fig. 2. (a) The ipsilateral PAM LWTs in gram force and (b) ratio of weight distribution between the hind limbs of sham ($n = 8$) and FCA-injected ($n = 8$) rats over a 28-day pilot study. PAM detected a large window of hypersensitivity, similar and comparable to that observed with the incapacitance tester. Statistical significance ($P < 0.05$) is represented by *; statistical analysis carried out to compare the two groups at each time point using a repeated measures ANOVA.

3.1.3. Joint inflammation

Basal measurements of joint diameter were 9.92 ± 0.08 mm for ipsilateral knees ($n = 16$), and 9.85 ± 0.12 mm for contralateral knees ($n = 16$). On day 1, the average ipsilateral knee joint diameter of FCA-injected animals was 13.11 ± 0.06 mm ($n = 8$), compared with 10.12 ± 0.1 mm ($n = 8$) in control animals. The swelling remained significant compared with sham animals on days 2, 5, 7 and 9 post-FCA ($P < 0.001$), however, thereafter the values returned towards basal levels, and no further significant differences were observed (data not shown).

3.2. Behavioural evaluation of the effect of a dose range of prednisolone, and single doses of morphine and celecoxib on the FCA-induced hypersensitivity

3.2.1. Pressure application measurement (PAM)

Measurements were made on days 0, 2, 4, 7 and 10, to determine that a window of hypersensitivity was present prior to drug treatment regimes. On each of these days the ipsilateral PAM LWTs of all FCA-injected animals ($n = 47$) were significantly less than those of the sham group ($n = 8$, $P < 0.0001$). On day 10, arthritic rats were randomly assigned into one of six treatment groups; celecoxib 15 mg kg^{-1} ($n = 8$), morphine 3 mg kg^{-1} ($n = 8$) or prednisolone 1 ($n = 7$), 3 ($n = 8$), or 10 mg kg^{-1} ($n = 8$). AUC values were calcu-

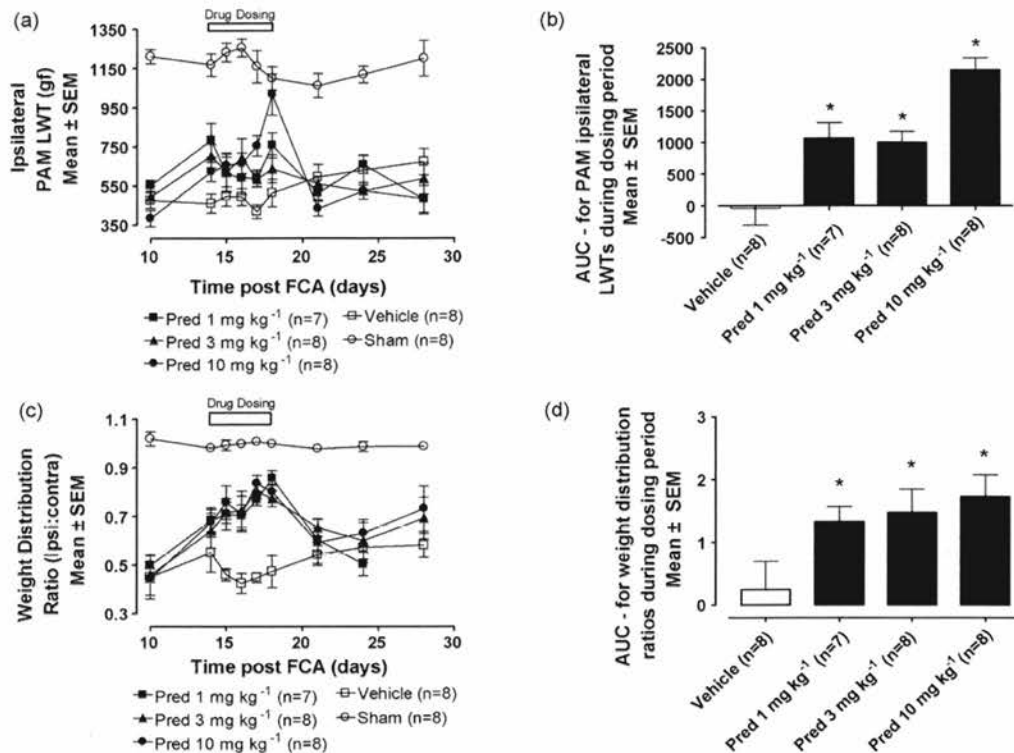


Fig. 3. The (a) ipsilateral PAM LWTs and (c) weight distribution ratios of vehicle and prednisolone treated arthritic rats and the corresponding graphs of AUC values for the two readouts during the dosing period (b and d). The drug-dosing period was between days 14 and 18 as indicated by the box on the graphs. The incapacitance tester was able to detect the dose-dependant reduction in hypersensitivity evoked by FCA in groups treated with 1, 3 and 10 mg kg⁻¹ prednisolone. PAM did not show a three-point dose-response, instead the 1 and 3 mg kg⁻¹ doses gave similar responses, whereas the 10 mg kg⁻¹ gave a greater reversal. Statistical analysis was carried out to compare the AUC values for the drug-treated group compared with vehicle; *Statistical significance ($P < 0.05$), determined by an ANOVA.

lated for each individual animal to observe any drug induced analgesia over the 5-day dosing period. AUC values were calculated using each animal's day 10 value as a baseline to observe changes from day 14 through to day 18. Results showed that PAM detected a significant analgesic effect of all drugs administered ($P < 0.0001$ for morphine, celecoxib and prednisolone 10 mg kg⁻¹, and $P < 0.05$ for prednisolone 1 and 3 mg kg⁻¹, compared with vehicle, see Figs. 3a and 4a).

Further PAM LWT measurements were taken after the cessation of drug-treatment, on days 21, 24 and 28. In all groups any analgesic effect of the compound previously administered was abolished, with there being no significant difference to the vehicle-treated group.

3.2.2. Incapacitance tester—weight distribution

Measurements were made on days 0, 2, 4, 7 and 10, to determine a window of hypersensitivity was present prior to drug treatment regimes. On each of these days the weight distribution ratio of all FCA-injected animals ($n = 47$) was significantly less than those of sham ($n = 8$, $P < 0.0001$). AUC values were calculated for the dosing period and showed that the FCA-induced hypersensitivity was significantly reversed by celecoxib 15 mg kg⁻¹ ($n = 8$), morphine 3 mg kg⁻¹ ($n = 8$) and prednisolone at 3 ($n = 8$) and 10 mg kg⁻¹ ($n = 8$) ($P < 0.05$, compared with vehicle), but not by 1 mg kg⁻¹

($n = 7$) prednisolone (see Figs. 3 and 4c). The weight distribution ratio AUC values also revealed a dose related reversal of hypersensitivity using the incapacitance tester (see Fig. 3d).

Further measurements made on days 21, 24 and 28, revealed that the incapacitance tester detected no lasting reversal of hypersensitivity following the cessation of drug administration with there being no significant difference between each drug group and vehicle ($P > 0.05$).

3.2.3. Joint inflammation

Subcutaneous dosing of 3 mg kg⁻¹ morphine or 15 mg kg⁻¹ celecoxib had no effect on FCA-induced joint swelling. However, prednisolone at 1, 3 and 10 mg kg⁻¹ all significantly reduced the average ipsilateral knee joint diameter, ($P < 0.05$, compared with vehicle) during the dosing period (data not shown).

3.2.4. Correlation of the PAM with weight distribution

In order to determine the strength of the correlation between the results obtained from the two readouts, a Spearman's linear regression analysis was carried out. The mean PAM ipsilateral LWT for each group was plotted against the mean ratio of weight distribution of the same group, with results from all experimental days included. A strong correlation between the

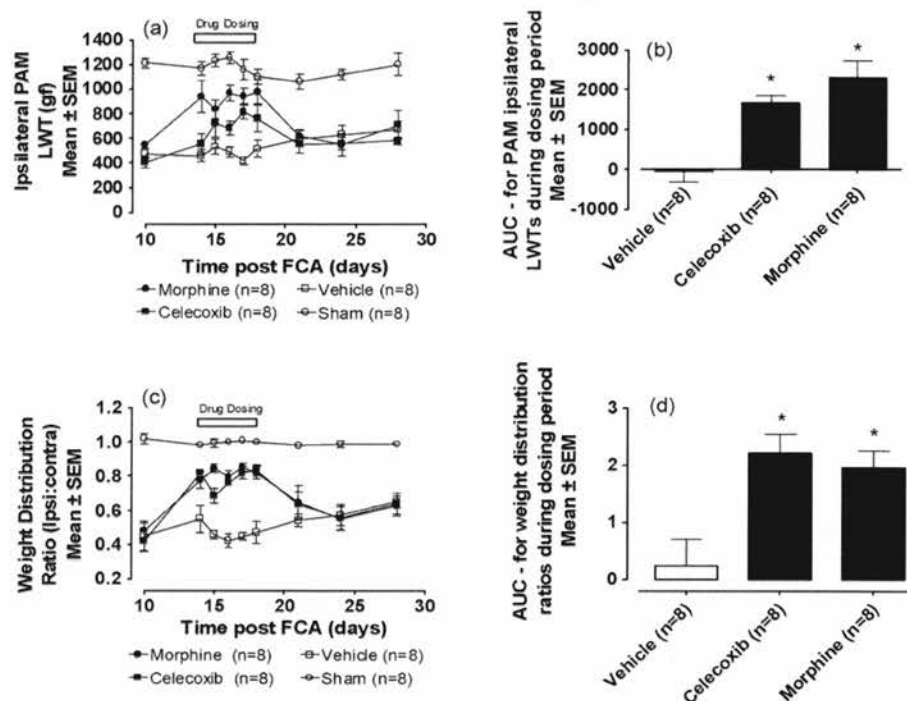


Fig. 4. The (a) ipsilateral PAM LWTs and (c) weight distribution ratios of vehicle-, morphine- and celecoxib-treated arthritic rats; and the corresponding graphs of AUC values for the two readouts during the dosing period (b and d). The drug-dosing period was between days 14 and 18 as indicated by the box on the graphs. PAM was able to detect the analgesic action of both drugs compared with vehicle. Statistical analysis was carried out to compare the AUC values for the drug-treated group to that of vehicle-treated animals using an ANOVA; *Statistical significance ($P < 0.05$).

results obtained from the two different readouts was observed (see Fig. 5a; Spearman $r = 0.91$; $P < 0.0001$).

3.2.5. Power analysis of rat drug study

The first power analysis performed (Fig. 5b) was calculated from the effect size equivalent to the observed difference between vehicle and the highest dose of prednisolone. Graphical results clearly show that in the structure of this experimental design, where each animal has three measurements taken for each readout, that a higher statistical power was achieved from the PAM readout compared with the weight distribution readout. In this experimental design if six animals per group were used for investigating a hypothesised drug effect equal to the observed effect, we could expect to achieve approximately 97% power using the PAM readout, but only 70% power with the weight distribution readout.

In a second power analysis (data not shown) results revealed that the statistical power could be greatly increased in the PAM readout, by increasing the number of measurements taken per animal from three to five, however increasing the number of animals used from 8 to 10 showed no real benefit. This was in contrast to the weight distribution readout, where increasing the number of animals from 8 to 10 greatly improved the power, but increasing the number of measurements showed no real benefit. This highlights the benefit of investigating the experimental design. For PAM we can actually increase the statistical power of an experiment by increasing the number of measurements while reducing the number of animals.

4. Discussion

This study has demonstrated the use of a novel behavioural tool for assessing joint pain in a rat model of experimental chronic joint inflammation. The results of these studies indicate that PAM provides a reliable, quantitative measurement of localised, FCA-induced mechanical hypersensitivity in the knee joint of rats. PAM was also able to detect the analgesic action of prednisolone, morphine and celecoxib. Furthermore, a strong correlation between the weight distribution readout and the PAM measurements were made, illustrating that PAM is a simple behavioural test that will be a valuable addition to current measures for assessing hypersensitivity in the joint.

Clinically, joint pain is assessed either subjectively using the Ritchie articular index or objectively using a dolorimeter (Langley et al., 1983; Ritchie et al., 1968). The Ritchie articular index allocates one of four grades of tenderness in patients' joints: "not tender" (0), "tender" (1), "tender and winced" (2) and "tender, winced and withdrew" (3). Previous studies carried out in this laboratory used a similar subjective scale of joint hypersensitivity (Gauldie et al., 2004). However, a quantitative measure, which mimics the pressure dolorimeter, would considerably improve assessments of experimental joint pain by providing an objective measure. The dolorimeter uses a gradually increasing force, applied in a perpendicular plane across the joint margin, to assess localised hypersensitivity of a human joint. The dolorimeter can be used on different joint types and has been adopted in clinical studies assessing osteoarthritis

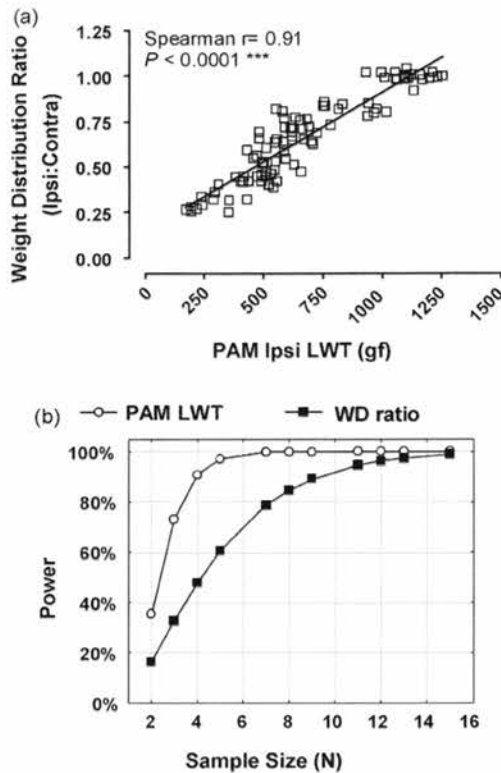


Fig. 5. (a) The correlation between the absolute ipsilateral LWTs (measured by PAM) and the ratio of the weight distribution between the hind limbs (measured by the incapacitance tester) in rats. There was a strong correlation between the two readouts (Spearman linear correlation factor, $r = 0.91$, $P < 0.0001$). (b) Analysis of the sample sizes required from the PAM and weight distribution (WD) readouts in triplicate measurements to reach certain statistical power levels. Results are calculated from an effect size equivalent to the observed difference between the vehicle group and the 10 mg kg^{-1} prednisolone group and results are based upon the observed variability in the study.

(Ottlinger et al., 2001) of the knee as well as the pain associated with fibromyalgia (Gracely et al., 2003). Currently, no objective measure of joint hypersensitivity is used to assess experimental arthritic joint pain in laboratory rodents. To that end, PAM was developed to align pre-clinical measures to those used clinically and help the translation of animal studies to human conditions.

Calibrated forceps have been used as a means of mechanical stimulation during electrophysiological recording (Gracely et al., 2003; Li and Neugebauer, 2004; Neugebauer and Li, 2002), or to measure knee joint or paw pain in experimental inflammation and primary hyperalgesia induced in deep tissues by inflammation (Han et al., 2005; Luis-Delgado et al., 2006; Yu et al., 2002). The latter involved direct application of the forceps to muscle or to the knee joint of rats (Cui et al., 1999; Skyba et al., 2005). However, although the technique is reported to be an easy to use, reliable method of assessing nociceptive withdrawal thresholds, they also show a steady increase in paw withdrawal threshold over a 9-day period that was highly correlated to an increase in weight gain over this time (Luis-Delgado et al., 2006). The authors suggest that the modification of pain sensitivity was due to increased size of the paw. This problem does not arise with PAM, as no change in pain threshold occurred

over a 3-week period, despite the animals gaining weight in each study. In the pilot study a significant increase in LWT was noted in ipsilateral joints of naïve rats and contralateral joints of all animals. This increase was due to the absence of initial induction measurements prior to the start of the study. Subsequently, a pre-study behavioural assessment was carried out prior to studying the effect of chosen gold standard analgesics, removing this initial anomalous result.

The authors in these studies reported several problems with the forcep style of device, including variation caused by the operator due to finger placement on the forceps, and inaccurate values of withdrawal thresholds, as the calibrated reading given is a function of the force applied by the experimenter and the resistance offered by the joint. PAM is calibrated directly across the force transducer and directly records the precise force placed across the knee joint, up until the point of joint withdrawal. As PAM uses a single force transducer worn on the experimenters thumb, the amount of operator variation will only occur in the placement of the transducer on the knee joint of the animal being tested. However this is only a minor issue as the large surface areas of the PAM thumb attachment removes potential inaccuracy in focusing on an exact point on the joint, which happens in the case of small forcep tips. A further advantage of PAM is the ability to change the size of the pressure application surface and the force range of the transducer, allowing the device to be used in several species as well as the possibility of assessing other joints and paws.

In this study, PAM proved to be an easy to use device, which allowed rapid, reproducible measurements, using triplicate measurements at each time point taken. The absence of a significant difference between the ipsilateral and contralateral LWT in naïve animals, together with the low variability, indicated that PAM could use the contralateral joint as an internal control, an idea originally proposed by Randall and Selitto (1957).

Typical withdrawal thresholds for naïve rats were approximately 1000 gf. These values are considerably higher than those seen in the paw using calibrated forceps or the Randall and Selitto device (Baamonde et al., 2004; Cook and Moore, 2006; Luis-Delgado et al., 2006; Randall and Selitto, 1957; Walker et al., 2003) which is probably due to the difference in the size and gross anatomy of the paw and the knee joint, in addition, the surface area over which the pressure is applied differs between the different devices. Pressure exerted onto the skin may activate nociceptive afferents in several tissues, depending on the surface area of the object used. Contact with a punctuate object such as a needle may exclusively activate nerve endings in the skin, in particular C fibres. Because deformation of the skin can be achieved with very small forces (Garell et al., 1996; Garnsworthy et al., 1988; Khalsa et al., 1997), these stimuli have little effect on afferents innervating deeper tissues. In contrast, a preferential activation of deep afferents is possible if pressure is exerted on a larger area of skin and the contact surface is rounded or padded (Treede et al., 2002). According to experiments using topical local anaesthesia, the contribution of cutaneous afferents to pain evoked by blunt pressure is minor (Kosek et al., 1995). This evidence adds support to the use of PAM as a measure of nociceptive activity in deeper tissues such as the joint itself,

rather than overlying skin. This is in contrast to von Frey hairs, which as a punctuate stimulus, activate skin afferents rather than those innervating the joint capsule and surrounding tissues.

Neugebauer and Li (2002) report that pressure stimuli $>50 \text{ g mm}^{-2}$ applied to the knee joint with calibrated forceps was noxious, as it consistently evoked hind limb withdrawal reflexes in awake rats. When values in our study were transformed into an approximate pressure the noxious LWT was $\sim 20 \text{ gf mm}^{-2}$. However, the data from PAM is not routinely presented as a force per unit area (pressure), as it cannot be assumed that the entire surface of the disc is in contact with the joint, furthermore in inflamed joints the deformation of the joint onto the disc is different to that in naïve animals. PAM measurements are therefore more accurately expressed as a force, in grams.

PAM was able to detect FCA-induced hypersensitivity, observed as a decrease in LWTs of around 60% compared with the basal levels in normal joints. This hypersensitivity was time matched to the significant inflammation observed following FCA injection. The ability of PAM to detect FCA-evoked hypersensitivity provides an excellent experimental model in which to test the analgesic properties of novel compounds over a period of stable inflammation between days 14 and 18 post-FCA.

Prednisolone, a standard positive control in drug screening (Pyne et al., 2004), was studied over a 5-day period to check whether the drug would lead to a resolution of FCA-induced hypersensitivity as a correlate to the hyperalgesia we would expect to see in man. PAM detected prednisolone analgesia, over the 5 days of dosing, which was abolished after dosing ceased. PAM also showed significant reversal of evoked mechanical hypersensitivity in arthritic rats treated with the opioid morphine and the cyclooxygenase-2 inhibitor, celecoxib, which was comparable to that seen in the weight distribution readout. Dose-related reduction of hypersensitivity to prednisolone was not observed using PAM whereas a dose related analgesia was observed using the incapacitance tester. When using weight distribution, the global effects of each drug on the whole animal are determined, whereas the PAM device is measuring a localised effect at the knee joint. This may explain why the PAM results observed were not dose related, as the doses were sub-threshold for a dose–response curve to be observed as measured by the PAM device.

When results are examined strong correlations are seen between the two behavioural assessments. These results suggest that both techniques have the ability to detect a joint hypersensitivity, which can be attenuated using gold-standard analgesics. PAM has the added advantage of assessing the hypersensitivity of the joint at the site of the inflammation, in a similar fashion to the clinical dolorimeter (Langley et al., 1983).

The 3R's play a large part in the planning of *in vivo* studies, where the aim is always to reduce the number of animals used, or refine the techniques involved. In this study we have shown that PAM measurements can be collected alongside the usual weight distribution recordings, increasing the information from a single set of animals. The power analysis performed on this study has shown that PAM has a higher statistical power as a tool for measuring the efficacy of drugs used to reverse the FCA-induced hypersensitivity than the weight distribution readout. The power

curves suggest that if PAM were used as a stand-alone readout in future studies then the number of animals per group could be reduced from eight to six, without compromising the statistical power of the study. Potentially PAM could be used to help reduce the number of animal's required in future joint hypersensitivity studies, but we are aware that these plots are based on the variability calculated from a single study. Replication of these results in further studies would increase the confidence in the conclusions drawn in this article.

In conclusion, the present study shows that PAM provides a novel, accurate behavioural tool for detecting a localised primary mechanical hypersensitivity in a rat model of chronic inflammatory joint pain. PAM is the first tool designed to measure both primary and secondary hypersensitivity objectively. It can be used in various experimental pain models and could be extended for use in other species. Results from these studies suggest PAM will aid the screening of novel analgesics designed to improve chronic inflammatory pain.

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Changes in the expression of Na_v1.7, Na_v1.8 and Na_v1.9 in a distinct population of dorsal root ganglia innervating the rat knee joint in a model of chronic inflammatory joint pain

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Abstract

Voltage-gated sodium channels play an essential role in regulating the excitability of nociceptive primary afferent neurones. In particular the tetrodotoxin-sensitive (TTX-S) Na_v1.7 and the tetrodotoxin-resistant (TTX-R) Na_v1.8 and Na_v1.9 channels have been suggested to play a role in inflammatory pain. Previous work has revealed acute administration of inflammatory mediators, such as Freund's complete adjuvant (FCA) or carrageenan caused an upregulation in the levels of Na_v1.7 and Na_v1.8 protein in DRG (dorsal root ganglia) tissue up to 4 days post-insult. In the present study, the expression of Na_v1.7, Na_v1.8 and Na_v1.9 was examined over a 28 day timecourse during a rat model of FCA-induced chronic inflammatory joint pain. Using the retrograde tracer Fast Blue (FB) and specific Na_v1.7, Na_v1.8 and Na_v1.9 sodium channel antibodies, immunohistochemical staining techniques were used to study sodium channel expression in a distinct population of L3–L5 knee joint afferent DRGs. In the ganglia, counts were made of positively labelled cells in the FB population. The results demonstrate that, following FCA injection, Na_v1.9 expression is upregulated at days 14, 21 and 28 post-FCA, with Na_v1.7 and Na_v1.8 showing increased channel expression at days 14 and 28. These observations are accompanied by a unilateral joint hypersensitivity in the FCA-injected knee indicated by a behavioural shift in weight distribution measured using an incapacitance tester. The increased presence of these channels suggests that Na_v1.7, Na_v1.8 and Na_v1.9 play a role, at least in part, in the maintenance of chronic inflammatory pain several weeks after the initial insult.

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Keywords: Sodium channels; Chronic inflammatory pain; Fast Blue; DRG

1. Introduction

Chronic inflammatory joint pain results, in part, from primary afferent fibres innervating the joint becoming sensitised through a series of complex peripheral mech-

anisms (Coggeshall et al., 1983; Hildebrand et al., 1991; Schaible and Grubb, 1993; Kidd and Urban, 2001). Voltage-gated sodium channels (VGSC's) are fundamental in regulating the excitability of neurones and significant changes in the expression of these channels can produce abnormal spontaneous firing patterns which can lead to chronic pain (Waxman et al., 1999; Lai et al., 2004).

A number of experimental models of inflammatory pain in the rat have revealed changes in the expression

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of various subtypes of VGSC within the dorsal root ganglia (DRG) during acute inflammation (Okuse et al., 1997; Tanaka et al., 1998; Black et al., 2004; Coggeshall et al., 2004; Gould et al., 2004; Villarreal et al., 2005). Of the nine sodium channel α -subunits (Goldin et al., 2000), a great deal of research has focused on the role that the tetrodotoxin-sensitive (TTX-S) $\text{Na}_v1.7$ and the tetrodotoxin-resistant (TTX-R) $\text{Na}_v1.8$ and $\text{Na}_v1.9$ isoforms, which are predominantly expressed in peripheral damage sensing neurones, play in inflammatory pain (Nassar et al., 2004; Priest et al., 2005; Ekberg et al., 2006). The expression of these channels in particular has been shown to be highly dynamic in models of both neuropathic and inflammatory pain and the modulation of these channels in DRG tissue may present a therapeutic opportunity for the selective manipulation of primary sensory neurones. Reports describe an increased expression of $\text{Na}_v1.7$ and $\text{Na}_v1.8$ in rat DRG neurones in models of chronic neuropathic and inflammatory pain (Amir et al., 2006; Ekberg and Adams, 2006), leading to enhanced neuronal excitability, whilst null mutant mice demonstrate attenuated response to inflammation-induced hypersensitivity and noxious stimuli (Akopian et al., 1999). The role of $\text{Na}_v1.9$ is less well understood, Priest et al. (2005) report no change in $\text{Na}_v1.9$ expression in DRGs in a mouse model of acute pain, but a potential role for the channel in the maintenance of chronic inflammatory pain has been suggested (Dib-Hajj et al., 1998; Tate et al., 1998; Fjell et al., 1999).

In the present study, we have examined the expression of $\text{Na}_v1.7$, $\text{Na}_v1.8$ and $\text{Na}_v1.9$ in ipsilateral L3–L5 DRGs at various time points throughout a model of FCA-induced, unilateral chronic inflammatory joint pain (Wilson et al., 2006; Martindale et al., *in press*). In order to focus the study on channel expression within the knee joint primary afferent population, the fluorescent retrograde tracer Fast Blue (FB) was injected through the intact skin and patellar ligament into the centre of the knee joint cavity (Kuypers and Huisman, 1984; Hanesch and Heppelmann, 1995). FB revealed the identity of cell bodies of neurones which innervate the knee joint, highlighting a specific neuronal sub-population of the DRG (Salo and Theriault, 1997; Ivanavicius et al., 2004; Hwang et al., 2005). DRG were stained using primary antibodies specific to $\text{Na}_v1.7$, $\text{Na}_v1.8$ and $\text{Na}_v1.9$ and counts of positively labelled cells within the FB population were made.

2. Methods

2.1. Animals

Experiments were performed in accordance with Home Office regulations and UK animal welfare guidelines and received Local Ethics Committee approval. All

experiments were performed using adult male Wistar rats weighing between 150 and 200 g upon arrival (Charles River, UK; $n = 54$). Animals were housed 4 to a cage in a 12 h dark/light cycle and were given access to water and standard animal feed *ad libitum*. All animals were allowed at least five days acclimatisation prior to behavioural testing.

2.2. Behavioural studies

An evaluation of how the animals distribute their weight across the hind limbs was measured using the rat incapitance tester (Linton Instruments, UK). The apparatus comprises two force transducers mounted side by side, capable of measuring the force placed through them over a set period of time. Animals were placed with each hind limb centred on a transducer platform and the force exerted on each transducer was measured over a 3 s period (Clayton et al., 1997; Wilson et al., 2006). The weight carried by the injected limb was divided by that carried by the un-injected limb giving a weight distribution (WD) ratio. A ratio of 1.0 results from the animal distributing its weight equally across both hind limbs. Each animal was measured three times on each test day and the mean weight distribution ratio (\pm SEM) was calculated for each group. Statistical analysis was carried out using a Kruskal–Wallis one way analysis of variance (ANOVA) followed by Dunn's post-hoc comparisons. *P* values less than 0.05 were considered statistically significant.

2.3. Retrograde labelling of dorsal root ganglia

In order to identify the cell bodies of afferent nerves innervating the knee joint, Fast Blue (FB, Illing Plastics, Germany) was used as a retrograde tracer to label articular DRGs (Hanesch and Heppelmann, 1995; Salo and Theriault, 1997; Catre and Salo, 1999; Ivanavicius et al., 2004). All rats ($n = 54$) were transiently anaesthetised using 3% isoflurane in oxygen and had their left (ipsilateral) knee joint shaved and cleaned using a dilute Hibiscrub solution. Using a 30-gauge needle (BD Microfine, USA) attached to a 100 μ l Hamilton syringe; 10 μ l of 2% FB was injected through the patella tendon into the joint space of the left knee. Animals were allowed to recover from anaesthesia before being returned to their cage, which was layered with soft paper bedding. Trial injections were performed on cadavers prior to the study to ensure that 10 μ l of FB could be injected into the knee joint cavity without visible leakage, minimising the chance that afferent cell bodies surrounding the knee would be falsely labelled.

2.4. Induction of chronic inflammatory hypersensitivity

All rats were transiently anaesthetised using 3% isoflurane in oxygen and had their left knee shaved and

cleaned using a dilute Hibiscrub solution. Rats in the Freund's Complete Adjuvant (FCA) groups ($n = 24$) were then injected with 150 μ l of FCA (1 mg ml⁻¹ *Mycobacterium tuberculosis*, Sigma, UK) through the patella tendon into the knee joint space using a 30-gauge needle (BD Microfine, USA). Control animals ($n = 30$) were untreated. Animals were allowed to recover from anaesthesia before being returned to their cage.

2.5. Study design

All animals ($n = 54$) were behaviourally assessed to determine the WD in naïve rats on day 0 prior to FB injection. Following the injection of the retrograde tracer, animals were further assessed for any change in WD at selected times over a 35 day period. On day 7 post-FB, animals were treated with FCA to induce a chronic inflammatory hypersensitivity, with control animals remaining untreated. Following behavioural assessments animals were killed in groups ($n = 6$) on days 7, 14, 21 and 28 post-FCA along with the corresponding time matched controls, and a single group of animals on day 0, using a rising concentration of CO₂ followed by cervical dislocation. Ipsilateral dorsal root ganglia (DRGs) were removed from levels L3, L4 and L5 and embedded in optimum cutting temperature (TissueTek, Canada) compound and snap frozen in isopentane cooled on dry ice ($n = 6$ per group). DRG were then stored at -80°C until they were sectioned.

2.6. Fluorescent immunohistochemistry

DRGs were sectioned using a Leica CM3050S cryostat. Sections were cut (12 μ m) and thaw-mounted in series on 10 slides (Superfrost Plus Gold, Menzel-Gläser, Germany) with each slide containing 5–8 sections at different levels throughout the DRG. Sections were fixed in 4% paraformaldehyde (30 min), washed with phosphate buffer solution (PBS; 2×5 min), and non-specific binding was blocked using a solution of 10% normal goat serum, 10% bovine serum albumin and 80% PBS (60 min). Sections were then labelled overnight (4 $^{\circ}\text{C}$) with affinity isolated rabbit immunoglobulins raised against unique synthetic peptides immunogens corresponding to the sodium channels Nav1.7 (K241,1:200), Nav1.8 (K106,1:200), and Nav1.9 (K186,1:200). These specific alpha sodium channel sub-type antibodies (GlaxoSmithKline, Harlow, UK) were all developed to cross-react with both human and rat antigens (Amaya et al., 2000; Coward et al., 2001a,b). Additional sections were incubated as described above, but with the omission of the specific primary antibody to act as negative controls. The following day, sections were washed in PBS (3×5 min) and sites of antibody labelling were revealed using the secondary antibody Alexa-488 goat anti rabbit (1:200, A11094, Molecular

Probes, UK; 120 min at 37 $^{\circ}\text{C}$). Sections were washed in PBS (3×5 min) and coverslipped using Citifluor solid mounting kit (CFPVOH and AF100, Citifluor, UK).

2.7. Data acquisition and analysis

For each antibody FCA-injected animals at days 7, 14, 21 and 28 post-FCA and their corresponding time matched control animals were investigated. Each group contained six animals from which ipsilateral L3–L5 DRGs were collected from each animal. Fluorescence in DRGs was observed using a Leica DMR microscope. Sections were initially observed under an excitation frequency of 380nm to reveal and count the total number of FB positive cells in 6–8 sections from each DRG. Setting the microscope filter to excite the sections at 488nm then enabled the sodium channel fluorescing cells to be visualised. By switching the filters between the two different excitation wavelengths the total number of double-labelled cells were identified and counted. From the total FB count and the double labelled count, the mean animal co-localisation percentage (\pm SEM) of sodium channel positive cells within the FB positive sub-population was calculated at each time point. Only cells with visible nuclei were counted in order to avoid counting positive cells more than once. All analyses were carried out with the observer blind to the groupings and representative photomicrographs were captured throughout. Statistical analyses were carried out using Mann Whitney tests \pm SEM, with statistically significant differences between groups confirmed as P values less than 0.05.

3. Results

3.1. Behavioural assessment of hypersensitivity

Intra-articular injection of FB alone had no effect on the mean weight distribution ratio (pre-injection vs. day 7 post-injection, ($n = 54$) 7 days post-injection, and at all time points throughout the control groups (Fig. 1). Injection of FCA resulted in the mean weight distribution ratio (\pm SEM) significantly decreasing from the pre-injection value of 0.96 ± 0.03 to a day seven post-FCA value of 0.37 ± 0.03 (Mann Whitney, $n = 54$, $P < 0.001$). FCA animals had a significantly lower weight distribution ratio at all time points post-injection when compared to control (ANOVA, $n = 54$, $P < 0.001$). There was no significant difference within the control groups at any time point.

3.2. Fast Blue fluorescence

Following the intra-articular injection of FB the fluorescent tracer was retrogradely transported by knee joint primary afferent nerves into their cell bodies in the

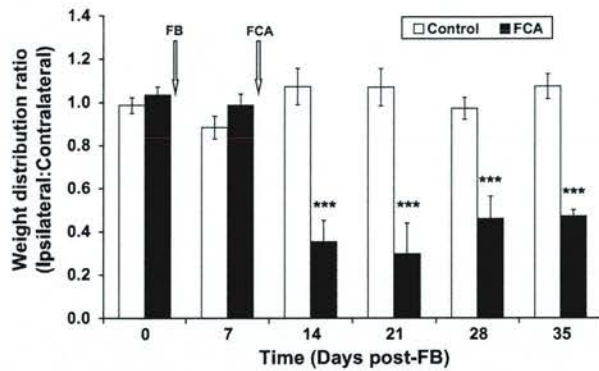


Fig. 1. A time course study ($n = 54$) of the weight distribution changes following injection of FB and FCA. Intra-articular injection of FB into the left knee joint caused no significant difference in the mean weight distribution (pre-injection vs. day 7 post-injection, $n = 54$). Intra-articular injection of FCA into the same knee seven days later caused a significant reduction in the weight distribution across the hind limbs at all time points post-FCA, as the rats place less weight through the ipsilateral limb. Kruskal–Wallis ANOVA followed by Dunn's post-hoc ($***P < 0.001$; FCA vs. control, $n = 6$; day 14 = 1.07 ± 0.08 vs. 0.35 ± 0.10 and day 35 = 1.07 ± 0.06 vs. 0.47 ± 0.03).

DRG. This resulted in an average of 7 (min = 0, max = 28) FB positive fluorescent cells being identified per individual ganglion (Fig. 2). These cells were thus identified as afferents from the knee joint.

A total number of 2463 FB positive cell bodies were counted during the study. There was no significant difference in the mean number (\pm SEM) of FB positive cells counted in FCA or control animals at each time point per group (82 ± 5 vs. 92 ± 6 ; Mann Whitney). Of the 2463 FB positive cells counted, 44% were found in L3, 38% in L4 and the remaining 18% in L5. Brightly fluorescent satellite cells surrounding FB-positive cell bodies were observed throughout the study at each time point.

3.3. Fast Blue and Na_V channel co-localisation

Fluorescent immunohistochemistry enabled both the sodium channel positive cells (green, Fig. 3b and e) and the FB population of cells (blue, Fig. 3a and d) to

be determined in the same sections. Absence of bleed-through between fluorescent filters was confirmed by the presence of three types of cell, namely cells that were only positive for one of the sodium channels, cells that were only positive for FB, and cells that were positive for one of the sodium channels and also for FB (Fig. 3c and f).

3.3.1. Changes in $Na_V1.7$ channel expression

The effect of FCA on $Na_V1.7$ expression in the FB positive L3–L5 ipsilateral DRG cell body population was assessed on days 7, 14, 21 and 28 post-FCA (Fig. 4). FCA caused a significant increase in the mean group percentage (\pm SEM) of $Na_V1.7$ positive cells at days 14 and 28 increasing from $32.4\% \pm 3.4$ to $45.9\% \pm 2.7$ and $25.0\% \pm 5.2$ to $43.5\% \pm 3.8$, respectively ($P < 0.05$, Mann Whitney, control vs. FCA; Fig. 5b and d). In control animals no significant change was observed in the group mean percentage at any time point over the 28 day period. A total of 406 FB-positive cells were counted in all control groups and 385 in all FCA groups. When all the time points of both FCA-injected and control animals were considered together, the mean percentage of $Na_V1.7$ positive cells in FCA-injected animals was significantly higher ($P < 0.01$, $42.2\% \pm 1.7$ vs. $32.3\% \pm 2.3$) than that in all the control animals.

3.3.2. Changes in $Na_V1.8$ channel expression

Following administration of FCA the expression of $Na_V1.8$ in FB positive cell bodies of ipsilateral L3–L5 DRGs was assessed on days 7, 14, 21 and 28 post-FCA (Fig. 4). Following injection of FCA there was a significant increase in the group mean percentage of $Na_V1.8$ positive cells at days 14 and 28, increasing from $38\% \pm 2.3$ to $54.6\% \pm 2.6$ and $40.8\% \pm 2.1$ to $53.6\% \pm 4.7$ respectively ($P < 0.05$, $P < 0.01$, control vs. FCA; Fig. 5a and d). In control animals no significant change was observed in the group mean percentage at any time point over the 28 day period. A total of 419 FB positive cells were counted in all control groups and 391 in all FCA groups. The mean percentage of all $Na_V1.8$ posi-

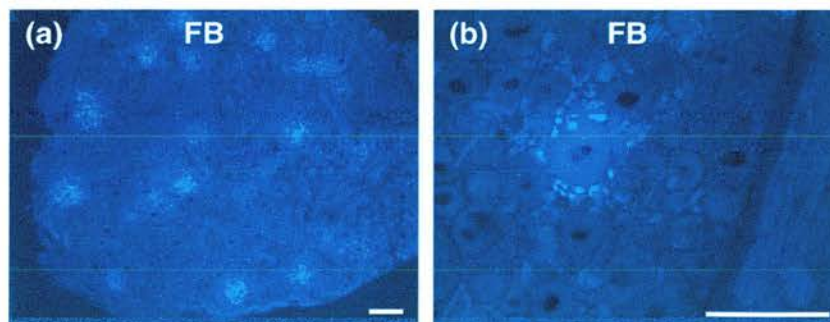


Fig. 2. Representative photomicrographs of FB fluorescence in ipsilateral L3–5 DRG tissue. (a) Note that only a small proportion of the total population of DRG cell bodies fluoresce and are easily identifiable from the non-fluorescing cells. Using a higher magnification (b) it can be seen that there are smaller satellite cells surrounding the fluorescing cells that are also brightly fluorescing. Scale bar = 100 μ m.

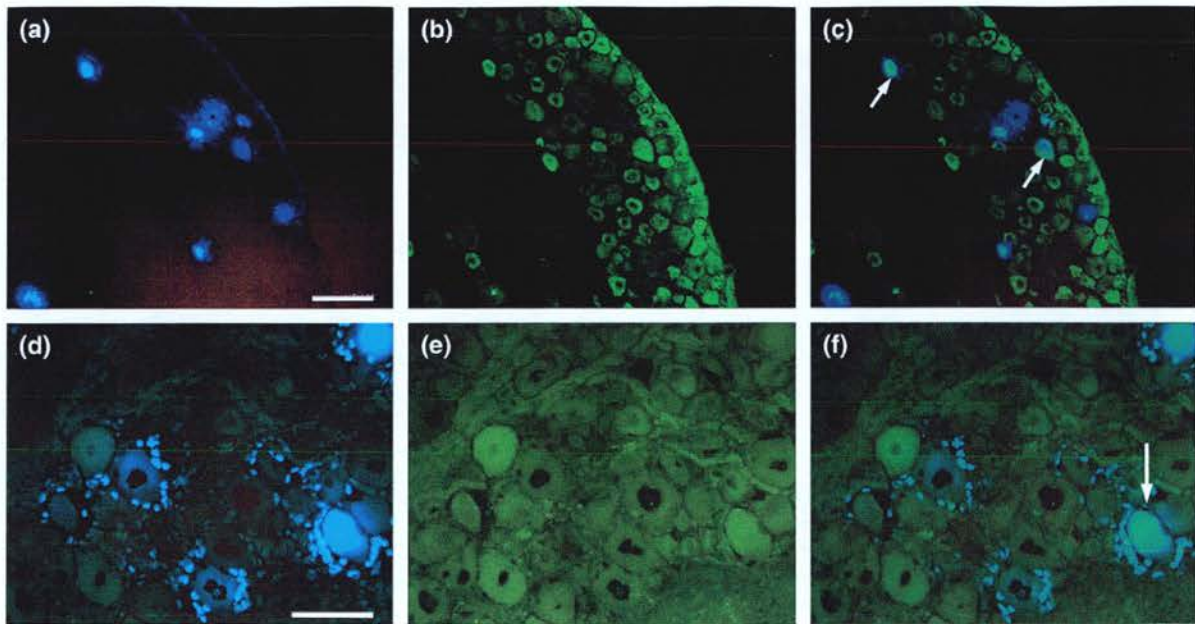


Fig. 3. Two rows of representative photomicrographs of two different sections (a–c and d–f). Images captured show (a and b) the FB fluorescence, (b and e) the NaV1.7 positive cells and (c and f) an overlay of the FB and NaV1.7 images. Arrows indicate cells positive for both the FB and NaV1.7 channel. Note that the FB positive cells (a and d) are not always necessarily positive for the sodium channel and visa versa. By switching between filters without moving the sections it is possible to count all the cells that are positive for both. Scale bar = 100 μ m.

tive cells at all time points in the FCA-injected set of animals ($49.8\% \pm 2.2$) was significantly higher ($P < 0.001$) than that in the control groups ($38.9\% \pm 1.1$).

3.3.3. Changes in NaV1.9 channel expression

Following administration of FCA, NaV1.9 expression within the FB positive population of L3–L5 ipsilateral DRGs was quantified on days 7, 14, 21 and 28 post-FCA (Fig. 4). Injection of FCA resulted in a significant increase in the group mean percentage (\pm SEM) of NaV1.9 positive cells at days 14, 21 and 28 increasing from $22.7\% \pm 3.1$ to $46.4\% \pm 3.3$, $26.2\% \pm 5.2$ to $47.0\% \pm 5.6$ and $25.4\% \pm 3.7$ to $47.8\% \pm 3.9$ respectively ($P < 0.05$, $P < 0.01$, control vs. FCA, Mann Whitney; Fig. 5a–c). No significant change was observed in the group mean percentage at any time point over the 28 day period in control animals. A total of 415 FB positive cells were counted in the control groups and 309 in the FCA groups. The mean percentage (\pm SEM) of NaV1.9 positive cells in all the FCA-injected animals ($45\% \pm 2.2$) was significantly higher ($P < 0.001$) than that in all the control animals ($24.7\% \pm 1.9$) when every time point was taken into account.

4. Discussion

The levels of the VGSCs NaV1.7, NaV1.8 and NaV1.9 increase during the course of a well established preclinical model of chronic joint pain. Injection of FCA into

the intra-articular space of the knee joint produces a localised inflammation leading to the establishment of a chronic joint hypersensitivity that is maintained up to 90 days post-FCA as previously described (Wilson et al., 2006). The current study examined time points up to 28 days, when there is a stable ongoing hypersensitivity with reduced levels of inflammation, symptomatic of chronic inflammatory-induced pain. We utilised the fluorescent dye FB to study channel expression in L3–L5 ipsilateral DRGs of neurones exclusively innervating the inflamed knee joint (Salo and Theriault, 1997). All of the animals used in this study had joint hypersensitivity measured using the weight distribution behavioural readout (Clayton et al., 1997; Barton et al., 2007).

This study is the first to use a retrograde tracer to demonstrate significant changes in the expression of VGSCs in specific L3–L5 DRG neurones innervating the rat knee joint, following intra-articular injection of FCA into the same knee. Previously published studies have highlighted changes in NaV1.7, NaV1.8 or NaV1.9 channel expression in rat DRG tissue following injection of FCA or carrageenan into the hind paw (Black et al., 2004; Nassar et al., 2004). However, these studies not only differ in the location and type of adjuvant injected, but also in the time course being studied, with expression levels typically investigated between one to four days post-insult (Tanaka et al., 1998; Gould et al., 1999; Black et al., 2004; Nassar et al., 2004). One study investigated the long-term effects of FCA on sodium

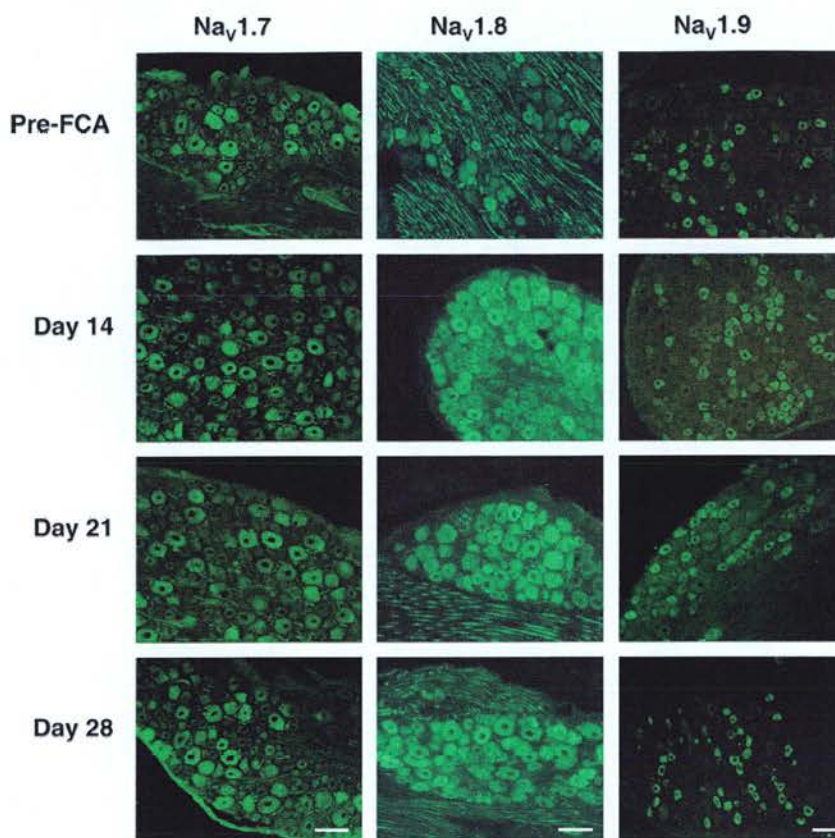


Fig. 4. Representative images of $\text{Na}_v1.7$, $\text{Na}_v1.8$ and $\text{Na}_v1.9$ immunofluorescence in FCA-injected ipsilateral DRG sections before administration of FCA and on days 14, 21 and 28 post-FCA. Note, that as only relatively few cell bodies in each image are FB positive (typically 2–5 per section) the VGSC immunofluorescence in all DRG cell bodies has been shown to demonstrate the subtle increases in channel expression during the time course. After administration of FCA it can be seen that $\text{Na}_v1.7$, $\text{Na}_v1.8$ and $\text{Na}_v1.9$ channel expression has increased on days 14 and 28 post-FCA, although it is less clear from simple visual inspection whether $\text{Na}_v1.7$ and $\text{Na}_v1.9$ expression is increased on day 21 post-FCA, highlighting the need for a quantitative count. Scale bar = 100 μm .

channel immunoreactivity in DRG, but used a pan sodium channel antibody that was unable to distinguish between the different channel isoforms (Gould et al., 1998). These results revealed a rapid increase in sodium channel expression 24 h post-FCA to levels that were maintained for at least 2 months, suggesting sodium channels play a critical role in the establishment and maintenance of chronic inflammatory pain.

Relatively few studies have investigated the role of $\text{Na}_v1.7$ in inflammatory hypersensitivity, but those that have report increases in channel mRNA and protein in L4/L5 DRG neurones four days after intra-plantar injection of carrageenan (Black et al., 2004), and L4–S1 DRG neurones 24 h post-FCA injection (Gould et al., 2004). Mutations in $\text{Na}_v1.7$ have recently been shown to occur in humans suffering from the painful inherited disease erythralgia (Waxman and Dib-Hajj, 2005), further suggesting the channel has the functional ability to play an important role in afferent pain signaling. In the present study intra-articular injection of FCA caused a significant increase in the number of

$\text{Na}_v1.7$ positive neurones within the distinct FB population of L3–L5 ipsilateral DRG on days 14 and 28 post-FCA. This is the first evidence that $\text{Na}_v1.7$ is upregulated in chronic models of inflammation and may indeed contribute to the maintenance of the FCA-induced joint hypersensitivity, which is sustained for weeks after the initial insult.

A much larger body of research exists surrounding the role of $\text{Na}_v1.8$ in the initiation and maintenance of chronic inflammatory pain (Amir et al., 2006; Ekberg and Adams, 2006). $\text{Na}_v1.8$ expression is rapidly upregulated in the early stages of inflammation following either a single injection of FCA or repeated injections of PGE_2 , suggesting the channel plays a key role in the initial induction of hypersensitivity (Gould et al., 2004; Villarreal et al., 2005). Support for a role of $\text{Na}_v1.8$ in the maintenance of inflammatory pain comes from studies reporting that there is a significant increase in mRNA in DRG tissue 4 days after carrageenan injection (Tanaka et al., 1998). Moreover, the increase in channel expression is likely to be associated with increased distri-

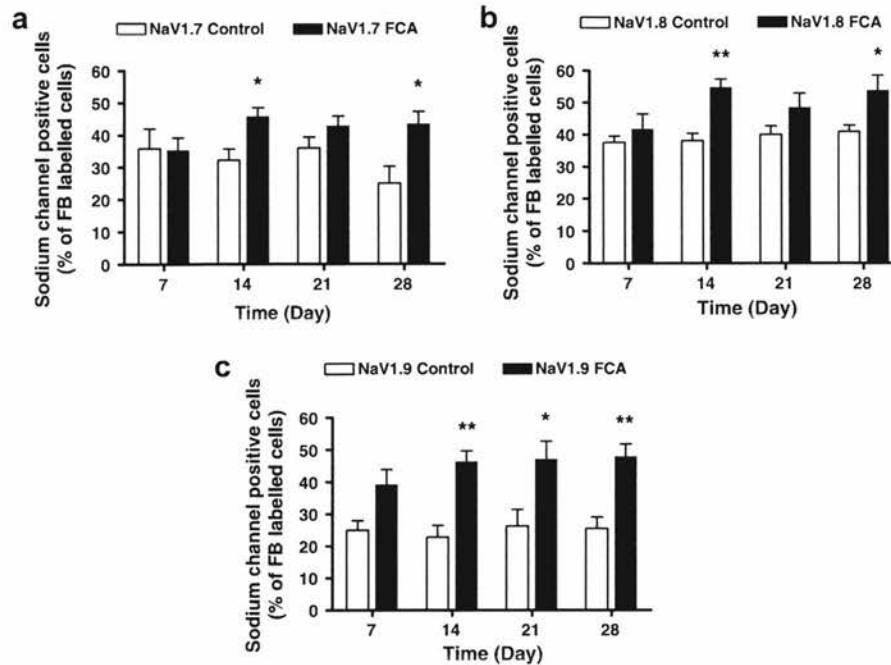


Fig. 5. Quantitative analysis showed that the percentage of: (a) NaV1.7, (b) NaV1.8 and (c) NaV1.9 immunofluorescent cells within the FB labelled DRG population are significantly upregulated in FCA treated animals, as compared with control animals at various points during the FCA timecourse (* $P < 0.05$, ** $P < 0.01$, FCA vs. control). Note that at 7 days post-FCA there is no significant difference in FCA treated animals and control animals with regards to all the NaV channels, and further no significant difference at day 21 when looking at NaV1.7 and NaV1.8 channels.

bution of the channels to peripheral nerve terminals as FCA-induced inflammation results in an increase in the amount of NaV1.8 channels observed in peripheral axons (Coggeshall et al., 2004). In the present study no significant increase in NaV1.8 was observed at the earliest time point, but there were significant increases in the expression of NaV1.8 within knee joint afferent neurones on days 14 and 28, in accord with the previously published studies suggesting a role for the channel in maintaining a chronic hypersensitivity.

Information on the role of NaV1.9 in inflammation-induced hypersensitivity is relatively scarce, with Black et al. (2004) reporting no change in the expression of NaV1.9 mRNA and protein in DRG four days after an intra-plantar carrageenan injection. However injection of FCA into the hind paw has been shown to increase DRG levels of NaV1.9 mRNA twofold, 7 days post-injection (Tate et al., 1998). Results from this present study show a significant increase in NaV1.9 channel expression on days 14, 21 and 28 post-FCA. Although NaV1.9 channel expression does not change in the early stages of inflammatory pain, and is therefore not implicated in the establishment of the hypersensitivity, we conclude its upregulation at later time points highlight a possible role for the channel in the maintenance of a chronic inflammatory pain.

The use of fluorescent retrograde tracers for tracking peripheral nerves through to their parent cell bodies is a well established technique (Kristensson,

1970; Kuypers et al., 1979; Kuypers and Huisman, 1984). Fast Blue can be transported over long distances, showing pronounced fluorescence in the cytoplasm of parent cells only 48 h after injection around the nerve terminals (Bentivoglio et al., 1980b). The use of fluorescent dyes *in vivo* to identify knee joint afferent cell bodies in DRG tissue is well described (Hanesch and Heppelmann, 1995; Ivanavicius et al., 2004). In this study 82% of the knee joint afferent cell bodies were found in L3 and L4 (data not shown), a result that concurs with previously published data (Salo and Theriault, 1997) using the retrograde tracer Fluoro-Gold where 88% of knee joint cell afferents were found in L3 and L4. There was no observable increase in the intensity of the fluorescence in cells of DRGs taken from animals with longer survival times, a trend that has previously been reported (Bentivoglio et al., 1980b). A phenomenon that was observed in the current study was the fluorescing of glial cells surrounding the retrogradely labelled cell bodies. This has been previously reported as being due to the diffuse migration of dye from the cell bodies into the adjoining satellite cells, and is thought to become more prominent the longer the dye is present in the animal (Bentivoglio et al., 1980a,b). In certain circumstances this might be of concern as it could potentially hinder the correct identification of positive cells within the DRG. However due to the intensely bright fluorescence of the cytoplasm in the present study we are confident,

that only those neurones with retrogradely labelled knee afferents were positively identified.

The significant increases in the expression of the VGSCs on days 14, 21 and 28 post-FCA observed in this study correlates with the presence of an FCA-induced joint hypersensitivity. A previous study using the same FCA model revealed significant increases in the levels of CGRP within all L3/L4 DRG neurones up to 35 days after intra-articular FCA administration, correlating with a behavioural hypersensitivity of that joint (Staton et al., 2007). It would be interesting to determine if this increase in CGRP expression takes place solely in the knee joint afferent population, or whether the injection has global effects within the DRG due to cross-talk between adjacent cells. Similar studies profiling changes in other key channels and receptors, such as the cannabinoids, opioids and the prostaglandins, which are known to play a key role in inflammatory hypersensitivity, may provide further insight into the multiple mechanisms involved in the establishment and maintenance of chronic inflammation-induced pain.

In summary, we have demonstrated a significant increase in the channel expression levels of the VGSCs; $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$ in a distinct group of ipsilateral L3–L5 DRG neurones during a model of FCA-induced, chronic joint pain. The sensitisation of primary afferent neurones in acute inflammatory pain has previously been reported to be due to changes in the expression of several VGSC's, although the process clearly involves considerably more than the modulation of just a single class of ion channels. Nevertheless, we successfully utilised a retrograde tracing technique to demonstrate that $Na_v1.7$ and $Na_v1.8$ are upregulated in afferent neurones specifically innervating the site of chronic inflammatory pain. Moreover, this study has increased our understanding of the neurobiology of inflammatory pain by revealing an increased expression in $Na_v1.9$ channels 14 days post-FCA highlighting a potential role of this channel in the maintenance of inflammatory hypersensitivity.

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